G32-634
#1

FIRST QUARTER PROGRESS REPORT
(AUGUST 21, 2000 - November 30, 2000)

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIONS: BEHAVIOR OF MICROORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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Submitted To:

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Edmonds, P., 1st Quarter Report, Dec. 1, 2000

Information provided in this "First Quarter Report" is a summary of some experimental work that is outlined in **Phase I. "Fundamental Microbial Taxonomic Analyses of Emulsions"** in the original proposal.

I. Personnel Assigned to this Project:

Paul Edmonds, Ph.D., Principal Investigator

Cindy Korir, Third-Year Ph.D. Student (Graduate Research Assistant)

Melissa Bowman, Second-Year M.S. Student (Expected to Graduate Dec. 16, 2000).

II. Initial Experiments.

The first five "contaminated" samples shipped from Air Products Polymers arrived September 8, 2000. These samples were stored at room temperature in the dark in my laboratory, Room 224, Cherry Emerson Building, 310 First Drive, on the Campus of the Georgia Institute of Technology, Atlanta, Georgia 30332. These samples are listed below in Table 1.

Our first **objective** was to analyze each of those samples for the "presence" of viable microorganisms (i.e., species intentionally added by Air Product's Personnel, and "natural contaminated samples). The **Spread-Plate Method** was used to analyze each sample for "total viable counts". Each sample was shaken well. Then, 10-ml was removed and serially diluted 10-fold in sterile 0.85% saline. Subsequently, 0.1 ml from each dilution was added to the surface of four different media (described below), and spread using a bend glass rod (hockey stick) to obtain isolated colonies. Plates were incubated at 37°C, and at room temperature. Then plates from each dilution that contained growth were observed for colony morphology at 24, 48, and 72 hours. Each "different" colony type was subcultured to an agar slant and stored.

Isolation Media Used When Analyzing Emulsion Samples in Table 1.

Trypticase Soy Agar (TSA) -- a general purpose medium for bacteria.

Phenylethanol Agar (PEA) -- a selective medium used for the isolation of staphylococci and streptococci, and is inhibitory to gram-negative bacteria.

Sabouraud Dextrose Agar (SAB) -- used for cultivation of fungi (yeasts and molds).

Psuedosel (Cetrimide Agar) Agar (PA) -- selective for the isolation of *Pseudomonas aeruginosa.*

Table 1. Contaminated Emulsion Samples

Label on Sample	Brief Description of Sample
1) Airflex 400 (Lot # F-145)	Intentionally contaminated with *Rhodoturula glutinis, Candida tropicalis, Candida guillermondi, Aspergillus* species, *Trichoderma virde, Sporothrix* species, *Cladosporium* species, and *Geotrichum candidum.*
2) Airflex 323 (Lot #A-147)	Intentionally contaminate with *Gluconoacetobacter liquefaciens.*
3) Arflex 323 (Lot # F-123)	Intentionally contaminated with a mixture of various bacteria.
4) Airflex 192 (Lot # D-66)	Intentionally contaminated (Same as No. 3).
5) Airflex 400H (Lot# B-118)	Naturally contaminated.

Results:

Table 2. Microorganisms Recovered (Based on Colony Type & Gram Stain Reaction)

Emulsion Type	Morphology & Gram Reaction
1) Airflex 400 (Lot # F-145).	
Pseudosel Agar:	No Growth of Microorganisms
Phenylethanol Agar:	Yeast (Slant # C_5 and Slant C_8)
Sabouraud Dextrose Agar	Mold (Slant # C_7 and Slant C_9)
Trypticase Soy Agar	Molds (Slant # C_1 and slant C_6), Yeast (Slant # C_2)
2) Airflex 323 (Lot # A-[illegible]47).	
Pseudosel Agar:	No Growth of Microorganisms
Phenylethanol Agar:	No Growth of Microorganisms
Sabouraud Dextrose Agar:	Yeast (Slant #$C_1$323 and Slant $C_4$323)
Trypticase Soy Agar:	Yeast (Slant #$C_3$323)
3) Airflex 323 (Lot # F-123).	
Pseudosel Agar:	No Growth of Microorganisms
Phenyethanol Agar:	Gram-Positive Rods (Slants #P_5, P_6, P_7, P_8, P_9)
Sabouraud Dextrose Agar:	No Growth of Microorganisms
Trypticase Soy Agar:	Gram-Positive Rods (Slants #P_1, P_2, P_3, P_4)

Table 2. (Cont'd)

4) Airflex 192 (Lot # D-66).

Pseudosel Agar: Gram-Negative Rods (Slants #M_{17}, D66 and M_{18} D66).

Phenyethanol Agar: Gram-Positive Cocci (Slants # M_7, D66, M_8, D66, M_9, D66, and M_{10} D66).

Gram-Positive Rods (Slants #M_{11} D66, M_{12} D66, M_{13} D66, and M_{14} D66).

Gram-Positive Cocci (slants # M_{15}, D66, M_{16} D66).

Sabouraud Dextrose Agar: Yeast (Slants # M_4 D66, M_5 D66, M_6D66).

Trypticase Soy Agar: Gram-Negative Rods (Slant # M_1 D66)

Gram-Positive Cocci (Slant # M_2 D66)

Gram-Positive Rods (Slant # M_3 D66)

5) Airflex #400H (Lot #B-118).

Pseudosel Agar: Gram-Negative Rods (Slants # M_1, M_2, M_3, M_4)

Phenylethanol Agar: Gram-Negative Rods (Slants # M_5, M_6, M_7, M_8)

Sabouraud Dextrose Agar: Gram-Negative Rods (Slants # M_9, M_{10}, M_{11})

Trypticase Soy Agar: Gram-Positive Cocci (Slant # M_{12})

Gram-Positive Rods (Slant # M_{13})

Summary/Discussion of Organisms "Recovered" from Emulsion Samples:

All organisms listed above that were recovered have been **subcultured** to Mueller-Hinton Broth + 15% Glycerol and stored in a Revco Freezer at -85°C. Subsequently, we can "activate" these organisms (when desired) for use in experiments and/or for **"Identification".** Please note that when it becomes "essential" for us to identify a particular organism, we can obtain this information "efficiently" by sub-contracting with a **commercial firm that will identify "isolates" on a fee/culture basis.**

As stated during your "Site Visit" to my laboratory at Georgia Tech (10/11/00) and in my follow-up E-Mail note (10/23/00), I have re-arranged **(in-part)** some of the **"initial"** experiments outlined in the Original Proposal to **focus on the "functional" role of organisms in the Emulsion Environment.** These experiments are divided into two groups:

(i) **The "Cross-Streak Interaction Experiment"** to determine if a "particular" organisms produces a substance **(antibiotic, bacteriocin, or lytic phage)** that will inhibit other organisms. Results from this group of experiment will provide "presumptive" information on "ecological succession" in the emulsion environment, and/or enable us to determine which species are genetically capable of becoming more "dominant" in the emulsion environment.

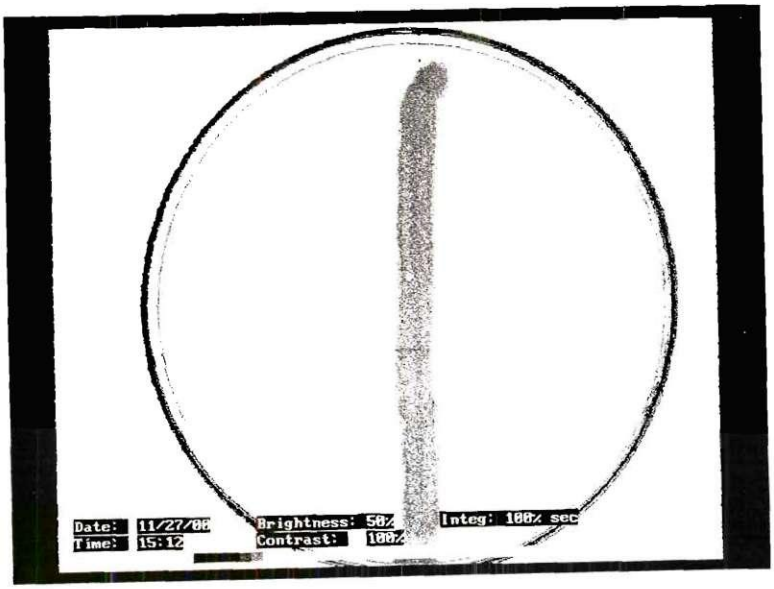
(ii) The other group of experiments will be designed to test the ability of organisms to **"Metabolize (Utilize or Degrade) Emulsion Components".** Briefly, a "chemically defined medium" (without any organic carbon or organic nitrogen) will be sterilized. After which, a measured quantity of a "designated" emulsion component will be added. Then, the vessel will be inoculated with a "Microbial species", and analyzed for **growth (increase in cell numbers).**

In an effort to utilize our time efficiently, and generate data from organisms interacting in the "Cross-Streak" experiment, we developed the following protocol utilizing a Yeast, as the presumptive "**Producer" organism.** This Yeast grew will on **"Sabouraud Dextrose Agar"**but not on the other media used in the **"isolation/recovery"** experiment, previously discussed.

I. Protocol for the "Cross-Streak Interaction Experiment":

A. One liter of Sabouraud Dextrose Agar was prepared, autoclaved, and poured into **"pre-sterilized glass petri plates"** (Note: Glass plates must be used, because one step in this experiment, require the use of **chloroform** [a reagent that will dissolve plastic petri plates]).

B. The presumptive **"Producer" organisms**" was cultivated in Sabouraud Dextrose Broth. From this broth culture, a sterile cotton swab was used to inoculate one Sabouraud Dextrose Agar plate, by making a **single streak** across the **center of plate** on the surface of the medium as shown below **(Figure 1),** after 72 hours of incubation at room temperature.



C. Following the 72-hour growth period, the glass-plate culture of the yeast was placed on the top of a laboratory bench in an **"inverted position"**.

D. Then, 0.5 ml of **Chloroform** was added to the **lid of the glass plate.** Immediately, the plate was closed**, and allowed to remain on the laboratory bench in the "inverted"** position for 30 minutes. (**Note: During this period, chloroform vapors denatures the lipids in the "cytoplasmic membrane" of the presumptive "producer organism" -- a lethal event**.)

E. Then, the residule cell debris (from the dead "producer organism") is removed with a sterile cotton swab, and discarded.

F. The same plate (containing) medium from which the presumptive "**Producer Organism"** had grown, is now **re-inoculated** by "**cross-streaking"** four different organisms, about one-inch apart **(horizontally crossing the area where "producer" organisms had grown)** on the surface as shown in the photo below. (**Note: Five Sabouraud Agar Plates [same producer strain] interacting against 20 different "cross-streaked" horizontally inoculated organisms constituted the first experiment).**

II. Results from this "preliminary" cross-streak experiment: Plate #5 is shown below (Figure 2), shows the "inoculated area" of four organisms, after the "secondary incubation period of 48 hours). Growth of organisms labelled "R" (a Gram-Positive Coccus) was inhibited by "substances" (antibiotics, bacteriocins, lytic pages, etc.) excreted into the medium by the "Yeast" during its growth period before being killed by chloroform vapors. Organisms labelled "Q" and "S" grew well on **Sabouraud Dextrose Agar,** but were not inhibited. **Organism labelled "T"** did not grow on **Sabouraud Dextrose Agar.**

PLATE NO. 5

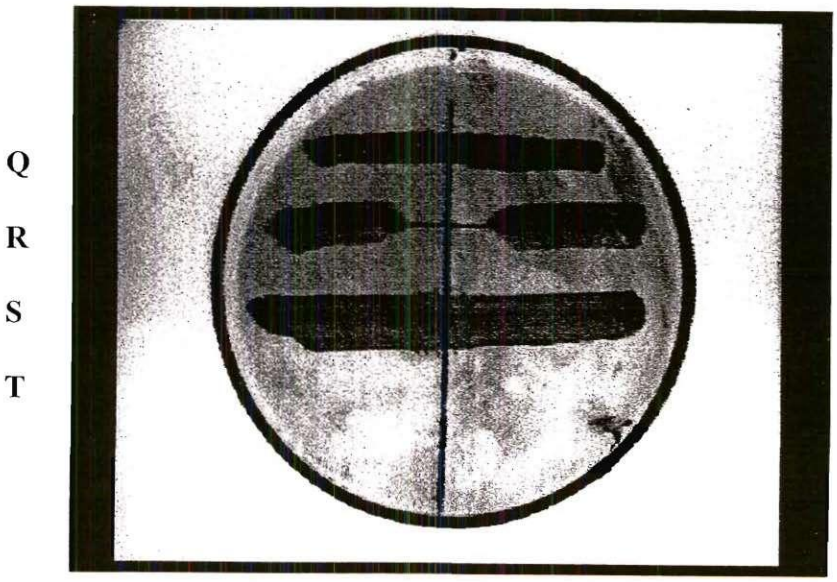


Figure 2. Plate #5 from the preliminary "Cross-Streak" Experiment. Areas of "cross-streaks" labelled "Q", "R", "S", and "T" represent specific organisms. Growth of organism "R" (**a Gram-Positive Coccus)** was inhibited by **"substances"** secreted into the medium by the **Producer (a Yeast).** Organisms "Q" and "S" grew well on Sabouraud medium, but their growth was not inhibited. Organism "T"did not grow on Sabouraud Dextrose Agar.

(NOTE: (i) Sabouraud Dextrose Agar was a medium "currently available" in my stock laboratory supplies. (ii) Subsequently, we tested all 20 organisms used in this experiment for their ability to grow on the following media: **Sabouraud Dextrose Agar (pH 5.6); Potato Infusion Agar (pH 6.8); and Potato Infusion Agar, supplemented with "Tartaric Acid"[pH 3.5]).** All of the 20 organisms (labelled A thru T) grew well on both Sabouraud Dextrose Agar, and on Potato Infusion Agar. **None grew on Potato Infusion agar, supplemented with Tartaric Acid.**

We have received the following **Emulsion Components (raw materials)**, and are beginning to test them in **Experiments utilizing "mineral medium - organic carbon and organic nitrogen)** to determine if organisms in Table 2 will grow (increase in numbers) and/or degrade them.

List of Raw Materials Received

Material	Brief Description
Igepal	C0-887
Igepal	C0-630
Colloid	675
Rhodapon	UB
Polystep	B-27
Natrosol	Type 250LR PA
Tergitol	(TM) 15-2-20
Airvol	523
Airvol	107
Airvol	205

Summary

Data and discussion in this report are "preliminary". These experiment will be repeated using both Sabouraud Dextrose Agar and Potato Infusion Agar. Furthermore, we have **ordered** the Type Strain of *Gluconoacetobacter liquifaciens* IAM 1834 (IFO 12388) from the American Type Culture Collection. This strain (in addition to the *Gluconoacetobacter* from your collection) will be the used in "controlled" cross-streaking experiments.

Simultaneously, we will continue to evaluate organisms that we have recovered from emulsion samples (those listed in Table 2).

Please E-Mail me your response to this report.

Signed: Paul Edmonds

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SECOND QUARTER PROGRESS REPORT
(December 1, 2000 - February 28, 2001)

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIONS: BEHAVIOR OF MICROORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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TABLE OF CONTENTS

EXECUTIVE SUMMARY

Information provide in this report pertains to the **"functional or ecological role"** of microorganisms in contaminated emulsions, and these data were generated from two categories of experiments: (I) cross-streak interaction experiments, and (ii) growth-challenge experiments to determine if "selected" species isolated from contaminated emulsions "metabolize (utilize) individual emulsion components".

Cross-Streak Interaction Experiments.

Twenty isolates of microorganisms (unknown bacteria, yeasts, and molds) were evaluated using the **"cross-streak interaction experiment"** to identify isolates that may exhibit a dominant role in contaminated emulsions. Each of these isolates were among species recovered in our laboratory from the five original emulsions samples obtained from Air Products Polymers (Airflows 400 [Lot # F-145]; Airflows 323 [Lot# A-147]; Airflows 323 [Lot# F-123]; Airflows 192 [Lot D-66]; and Airflows 400H [Lot #B-118]).

Preliminary results from a single "Cross-Streak Interaction Experiment" were shown in our **First Quarter Report** with organisms growing on Sabouraud Dextrose Agar. Prior to repeating that experiment, we tested all 20 organisms for their ability to grow on different formulations of Potato Dextrose Agar: (a) **Typical Formula** (potato infusion, dextrose, and agar [Remel, Lenexa, KS]), and (b) **Modified Formula** (potato infusion, dextrose, agar; supplemented with **"tartaric acid"** [Becton Dickinson, Sparks, MD]). All of the 20 isolates used grew well on **Potato Dextrose Agar (Typical Formula)**, and this medium was used to generate data contained in this report. Results obtained from these experiments (each performed in

duplicate) provide presumptive evidence that **three different species** (each designated as **"producer-organisms"**) excreted substances into the medium (Potato Dextrose Agar) that subsequently **inhibited** the growth of other isolates when inoculated on the same medium. These results suggest that each **"producer organism"** may exhibit a "**dominant role"** during ecological interactions in a contaminated emulsion. It is important to remember that the three "producer organisms" were selected "arbitrarily". We planned to evaluate ***Gluconacetobacter liquefaciens*** ordered from the American Type Culture Collection (ATCC 14835) for the "dormancy role", but it arrived February 16, 2001, **and** was not viable . (ATCC has promised a replacement viable culture). We do not know the nature of those excreted inhibitory substances. Each of the producer species (a yeast, and two different kinds of bacteria) has been preserved in Muller-Hinton Broth + 15% glycerol and stored at -85°C in a Revco Freezer. In later stages of this research project, any of these culture can be "activated by subculturing" (if desired), and used in other kinds of experiments designed specifically to characterize those inhibitory substances (i.e., determine if they are **bacteriocins, lytic phages, antibiotic, or other molecules).**

Growth-Challenge Experiments.

We evaluated the Yeast **"Producer-Organisms"** for its ability to grow in a mineral salts medium; supplemented with four separate emulsion components (Igepal C0-630; Colloid 675; Airvol 523, and Airvol 205). After an incubation period of two weeks at room temperature, no grow was observed in any experiments with emulsion components.

OBJECTIVES OF EXPERIMENTS COMPLETED IN THIS REPORT

Our specific objectives for experiments completed during the period covered in this report are stated below under the "general name" for each experiment.:

A. Cross-Streak Interaction Experiments.

Objectives:

(1) To determine an appropriate growth medium for use in all subsequent experiments.

(2) To perform each experiment with a single "producer" at least two times under the same set of evironmental conditions.

(3) To generate data that will demonstrate "domance" for a Producer-Organism, on the basis of its ability to excrete substances that will inhibit the growth of another organism.

B. Growth-Challenge Experiments.

Objectives:

(1) To test "Producer Organisms" for its ability to Utilize (Metabolize) a specific emulsion component "added" to mineral salts medium, and incubated at room temperature.

(2) To evaluate the "outcome" by measuring growth (change in population size), by using the Spread-Plate Method.

METHODS/PROCEDURES/PROTOCOLS

I. Protocol for the Cross-Streak Interaction Experiment.

A. Materials & Reagents.

1. Glass petri plates (one/4 test organisms)
2. Pre-sterilized cotton swabs
3. 1-ml pipets
4. Chloroform
5. Potato Dextrose Agar and Potato Dextrose Broth (Typical Formula), dispense the broth into tubes prior to autoclaving. Autoclave the agar in flask.

B. Procedure.

1. Prepare media according to manufacturers directions.
2. After sterilization of the Potato Dextrose Agar in an autoclave, allow to cool in a water bath set at 55°C.
3. Then, pour the medium in Pre-autoclaved plates. After solidification, incubate the plates overnight to detect any contamination that may have occurred when pouring the plates.
4. Use a sterile cotton swab to inoculatin the surface of potato dextrose agar using a Potato Dextrose broth culture of the "Producer organism" by making a "single" vertical streak.
5. Incubate these plates at the temperature appropriate for growth of the "Producer Organism".
6. At the end of the growth period, place each of the plates that contain the "Producer organism" on the laboratory bench in an "inverted postion."

7. Then, add 0.5ml of Chloroform to the inverted "lid" of each culture, and close the plate immediate. Allow the culture to set exposed to chloroform for 30 minutes.

8. Then, turn the plate to "top-side" up, and use a sterile cotton swab to remove "residual" growth (dead cells) killed by chcloroform vapor-disruption of cytoplasmic membrane.

9. Mark the outside edge of the plate for the "location" of previous growing Producer Organism (i.e., the vertical streak).

10. Select four potato dextrose broth cultures of organisms to be inoculated; each in a **single cross-streak** about one inch apart (horizontally crossing the area where the producer organism had grown).

11. Incubate all of these plates containing the cross-streaked organisms (secondary incubation period).

12. Following the secondary, growth period, record Inhibition (zone of no growth) over the area where the "producer organism" had grown.

13. Take photographs of all plates to make permanent record of experimental results.

II. Protocol for the Growth-Challenge Experiments.

A. Use a commercial source of "mineral Salt medium" such as **Bushnell-Hass Broth**, (Becton dickinson, Sparks, MD), and prepare according to manufacturers direction.

B. Then, dispense into bottles (each cotaining 90ml), and sterilize by autoclaving.

C. When cool, use a sterile 10 ml pipet to remove 5ml of the mineral medium to a separate sterile tube (use for culture suspension).

D. Use a separate sterile 10 ml pipet to remove 10 ml (or 10 gms if solid) of the appropriate **"emulsion component"**, and gently suspend in the mineral salts medium.

E. Now, suspend the appropriate concentration (pre-washed cells/ml) of the "producer strain in the 5ml of mineral salts medium (pre-drawn) in item (C, above).

F. Add this cell suspension (**the inoculum)**, which will consitute 100ml (including 10ml of the designated emulsion component).

G. From this culture, prepare 10-fold dilutions through 5 serial dilutions, using a separate pipet for each dilution.

H. Following the completion of the dilution series, use separate 1ml pipets to remove 0.1ml from each tube, and add it to the center of a potato dextrose agrar plate (these can be plastic). **Immediately, dip the hockey stick into alcohol, flame to sterilize, cool and "spreak the culture" evenly on the surface by rotating the plate.**

I. Incubate all plates at the temperature appropriate for the organisms.

J. Incubate the Culture in F (above), and **repeat "G" through "I" for each time period in the experiment (0hr through XHr).**

K. After the inbation period, remove the plates and count the colonies/plate/dilution. **Only plates that contain between 30 and 300 colonies are valid for calculating the number of colony forming units (CfU X the dilution factor) to yield CFU/ml.**

RESULTS

Interactions with the **Yeast "producer strains" (C_3323T_2SAB)** are shown in Figures 1 to 5.

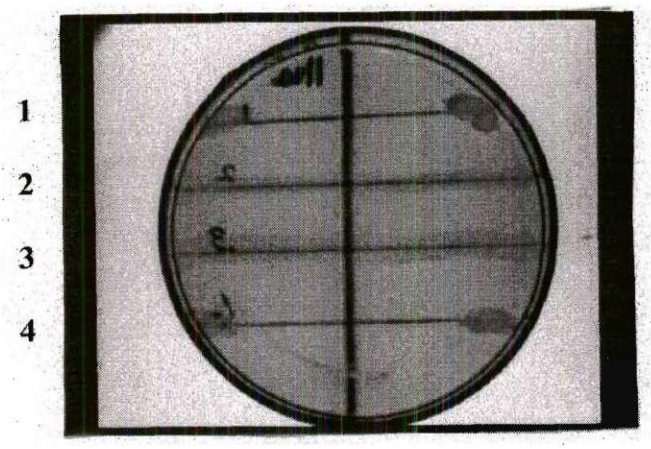

Fig. 1. Cross-streaked organisms are: 1) $M_{3b}192D66T_1TSA$ (+), 2) M_6400HT_1PHE(-), 3) $M_{b7a}400HT_1PHE$, 4) $M_{3a}192D66T_1TSA$. Inhibition = (+), No Inhibition = (-).

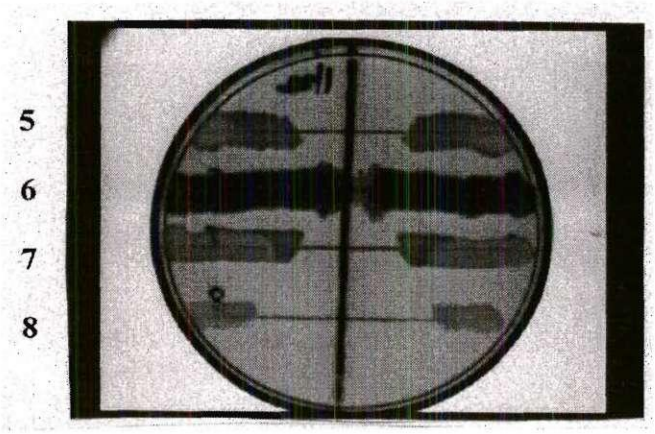

Fig. 2. Cross-streaked organism are: 5) $M_{11}192D66T_2TSA$ (+), 6) $M_{14}192D66T_1PHE$ (+/), 7) $M_{15b}192D66T_1PHE$ (+), 8) $M_{3a}192D66T_1TSA$ (+). Inhibition = (+), No Inhibition (-).

Yeast "Producer-Strain" Results (Cont'd).

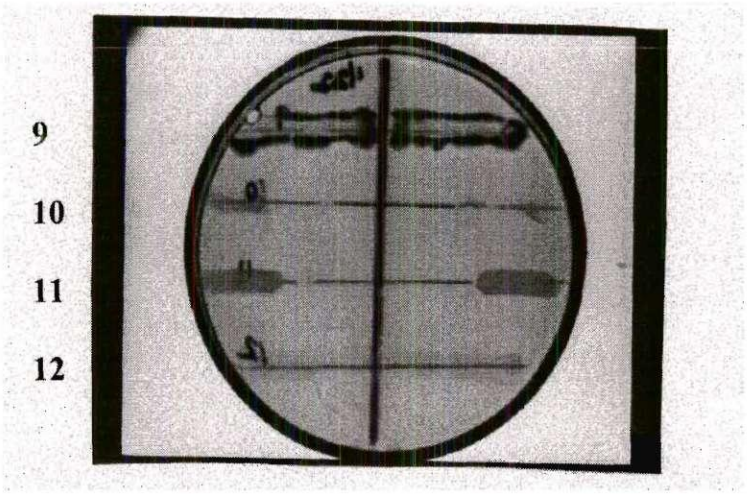

Fig. 3. Cross-streaked organisms are: 9) $M_8192D66T_2$ SAB (-), 10) $M_{17}192D66T_1$PSEU (+), 11) $M_5192D66T_2$PSEU (+), 12) M_9400T_2PHE (+). Inhibition: (+), No Inhibition: (-).

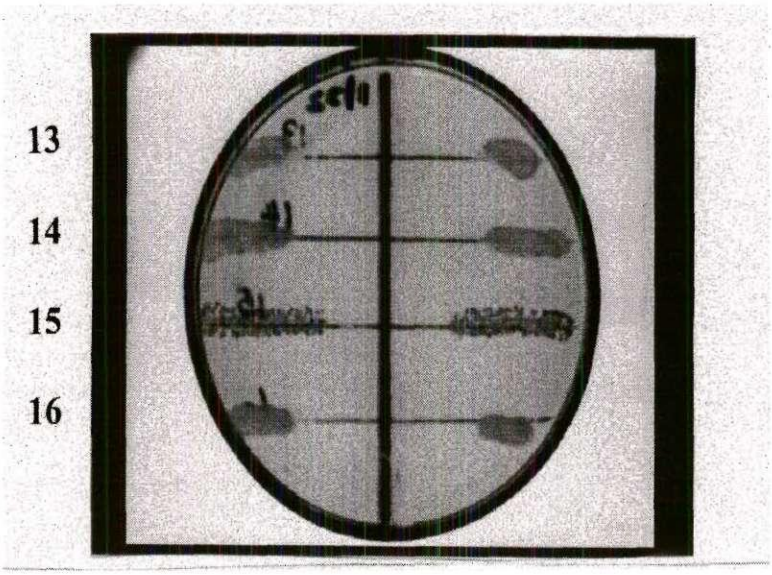

Fig. 4. Cross-streaked Cultures are: 13) $M_{18b}192T_1D66T_1$PSEU (+), 14) $M_{18a}192D66T_1$PSEU (+), 15) $M_{10b}192D66T_1$PHE (+), 16) $M_9192D66T_1$PHE (+). Inhibition: (+), No Inhibition: (-).

Yeast "Producer-Strain" Results (cont'd).

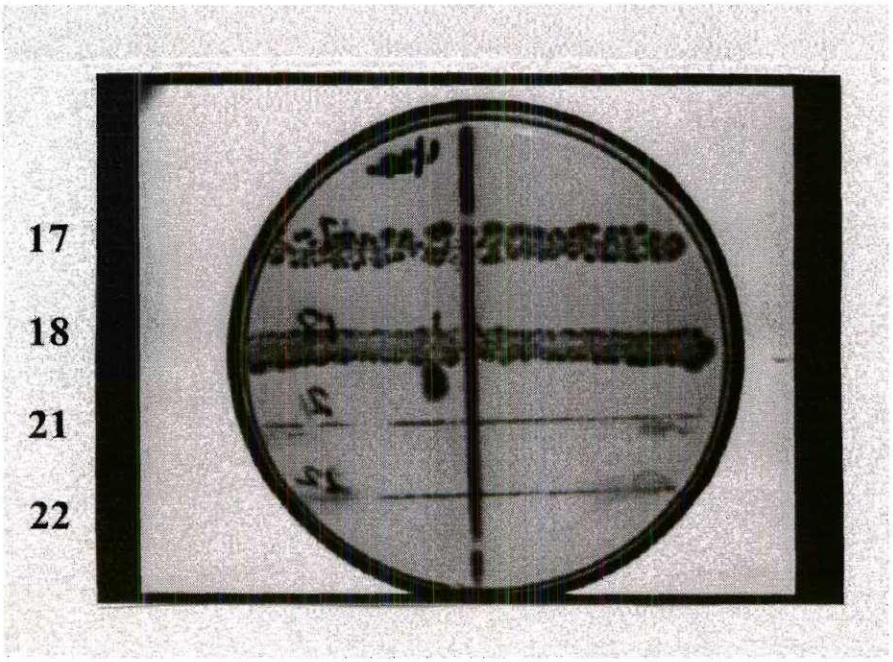

Fig. 5. Cross-streaked organisms are: 17) $M_4$192D66T_1SAB (-), 18) M_{19b}192D66T_1TSA (-), 21) $M_2$192D66T_1 TSA (+), 22) M_{16}192D66T_1PHE (+). Inhibition: (+), No Inhibition: (-).

Interactions with **"Producer-Strain ($M_1$192D66T_2TSA)** are shown in Figures 6 to 10.

1

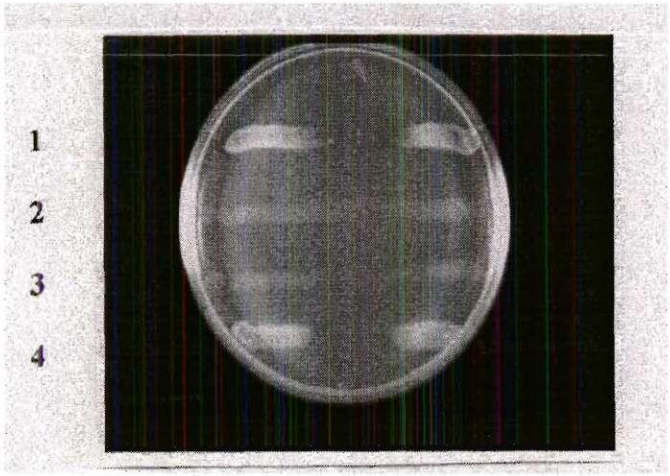

Fig. 6. Cross-streaked organisms are: 1) M_{3b}192DT_1TSA (+), 2) $M_6$400HT_1PHE (+), 3) M_{b7a}400HT_1PHE (+), 4) M_{3a}192D66T_1TSA (+). Inhibition: (+), No Inhibition: (-)

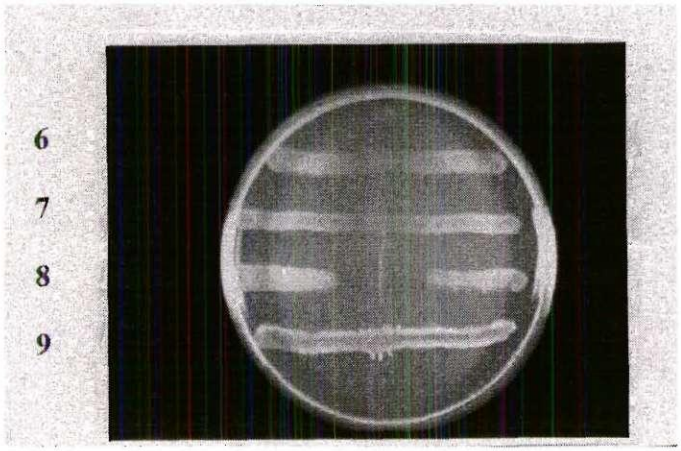

Fig. 7. Cross-streaked organisms are: 6) M_{14}192D66T_1PHE (-), 7) M_{15b}192D66T_1PHE (-), 8) M_{3a}192D66T_1TSA (+), 9) $M_8$192D66T_2SAB (-). Inhibition: (+) , No Inhibition: (-).

Bacterial "Producer-Strain" Results (cont'd)

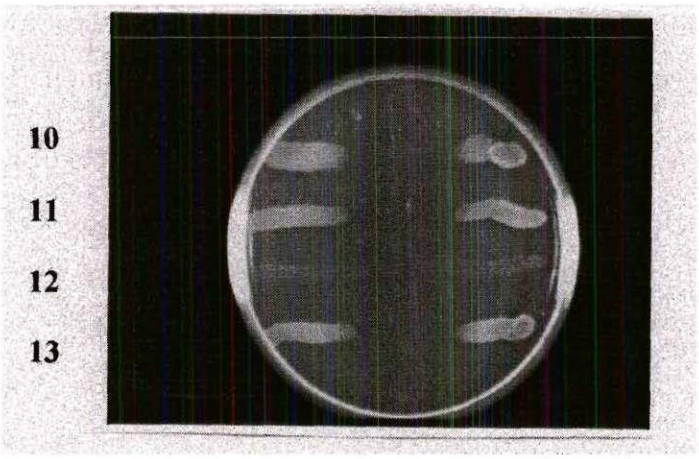

Fig. 8. Cross-streaked organisms are: 10) $M_{17}192D66T_1PSEU$ (+), 11) $M_5192D66T_2PSEU$ (+), 12) M_9400HT_2PHE (+), 13) $M_{18b}192D66T_1PSEU$ (+). Inhibition: (+), No Inhibition: (-).

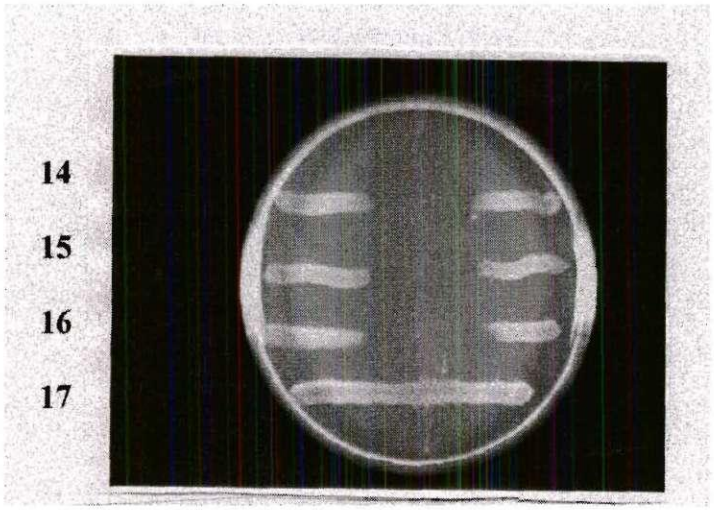

Fig. 9. Cross-streaked organisms are: 14) $M_{18a}192D66T_1PSEU$ (+), 15) $M_{10b}192D66T_1PHE$ (+) 16) $M_9192D66T_1PHE$ (+), 17) $M_4192D66T_1SAB$ (-). Inhibition: (+), No Inhibition: (-).

A Bacterial "Producer-Strain" Results (cont'd)

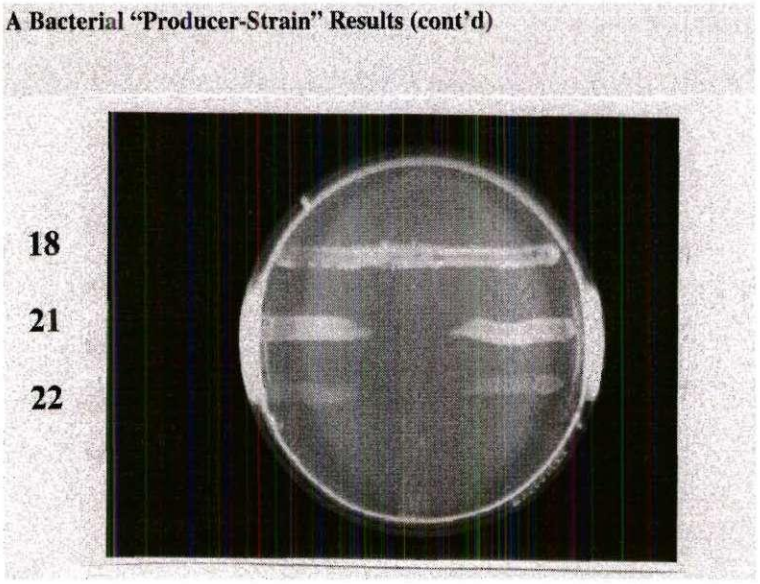

Fig. 10. Cross-streaked organisms are: 18) M_{19b}192D66T_1TSA (-), 21) $M_2$192D66T_1TSA (+), 22), M_{16}192D66T_1PHE (+). Inhibition: (+), No Inhibition: (-).

Interactions with **"Producer-Organism" ($M_9$400HT$_2$Phe)** are shown in Figures 11 to 15.

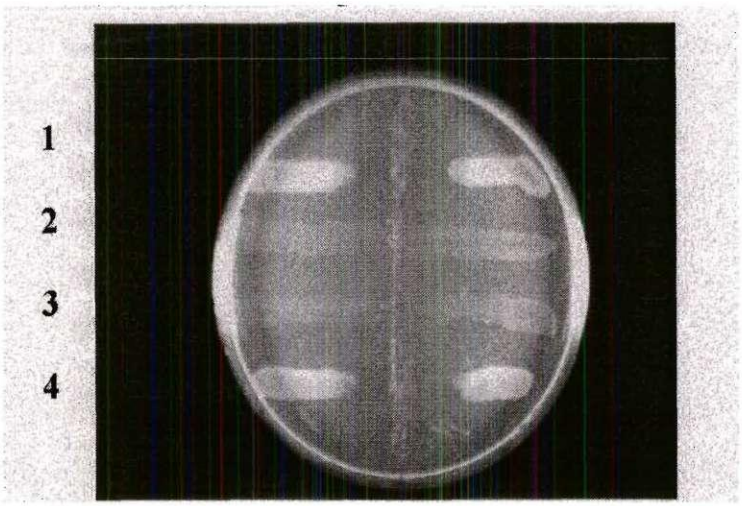

Fig.11. Cross-streaked organisms are: 1) M_{3b}192D66T$_1$TSA (+), 2) $M_6$400HT$_1$PHE (+), 3) Mb_{7a}400HT$_1$PHE (+), 4) M_{3a}192D66T$_1$TSA (+). Inhibition: (+), No Inhibition: (-).

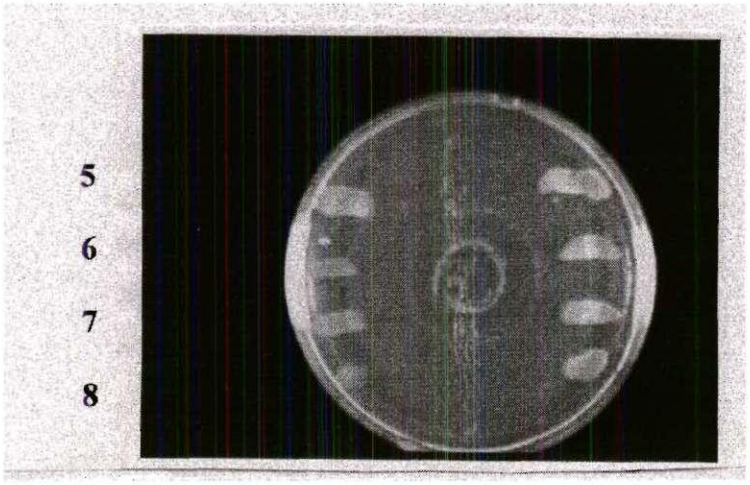

Fig. 12. Cross-streaked organisms are: 5) $M_1$192D66T$_2$TSA (+), 6) M_{14}192D66T$_1$PHE (+), 7) M_{15b}192D66T$_1$PHE (+), 8) M_{3a}192D66T$_1$TSA (+). Inhibition: (+), No inhibition: (-).

Bacterium Producer-Strain (M_9400HT_2Phe) Results (cont'd)

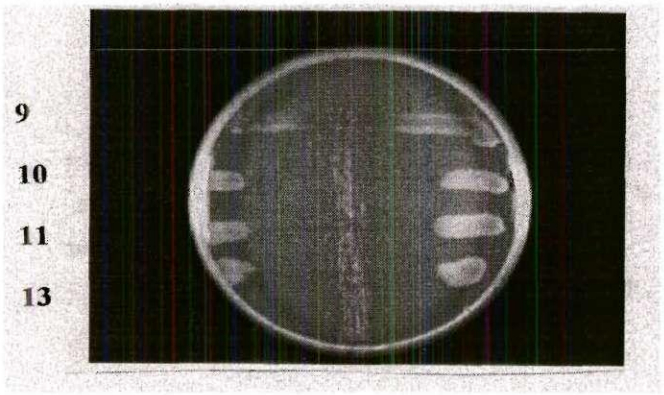

Fig. 13. Cross-streaked organisms are: 9) $M_8192D66T_2SAB$ (+), 10) $M_{17}192D66T_1PSEU$ (+), 11) $M_5192D66T_2PSEU$ (+), 12) Organisms Omitted, 13) $M_{18b}192D66T_1PSEU$ (+). Inhibition: (+), No Inhibition: (-).

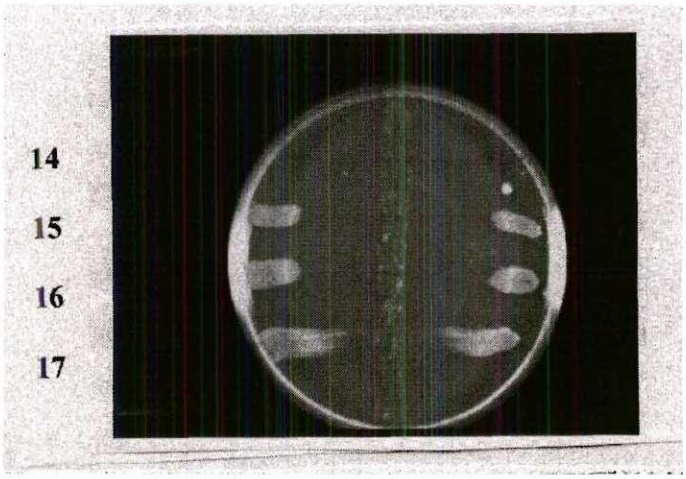

Fig. 14. Cross-streaked organisms are: 14) $M_{18a}192D66T_1PSEU$ (No Growth), 15) $M_{10b}192D66T_1PHE$ (+), 16) $M_9192D66T_1PHE$ (+), 17) $M_4192D66T_1SAB$ (+). Inhibition: (+), No Inhibition: (-).

Bacterium Producer-Strain ($M_9$400HT$_2$Phe) Results (cont'd)

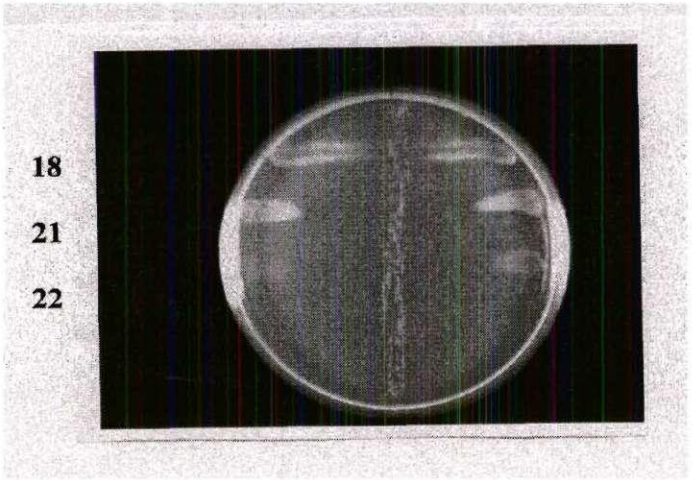

Fig. 15. Cross-streaked organisms are: 18) M_{19b}192D66T$_1$TSA (+), 21) $M_2$192D66T$_1$TSA (+), 22) M_{16}192D66T$_1$PHE (+). Inhibition: (+), No Inhibition: (-).

It is important to note that the numerical numbers for **cross-streak** organisms in each of the experiment with different **producer-strains** were not sequential. Cultures numbers "19" and "20" were omitted because their growth characteristics were not reproducible at the time these experiments were set up. We do not know the nature of the inhibitory substances, but all organisms and **producer strains** have been stored in Mueller-Hinton + 15% Glycerol at -85°C.

Results for Growth- Challenge Experiments.

We tested the Yeast "Producer-Organism" for its ability to grow in a Mmineral Salt Medium supplemented with Emulsion Components (Igepal C0-630; Colloid 675; Airvol 523 and Airvol 205), at a 10% concentration. The growth medium was inoculated with washed cells and incubated at room temperature. Growth measurements were determined by the Spread-Plate method at 24, 48, 72 hours, and 14 days. The Yeast culture did not grow (i.e., increase in cell numbers) at the end of 14 days.

INTERPRETATION AND SIGNIFICANCE

The results from experiments carried out during the period covered in this report are significant, because these data suggest that three **Producer-Organisms** (a Yeast and two different bacterial species) may exhibit a domance role in contaminated emulsions by inhibiting other species. In all habitats, microorganisms exist in mixed populations and ecosystems are dynamic because different species **inhibit** other species and/or compete for resources.

It is important to mentioned that our work was undertaken with organisms isolated in our laboratory from emulsion samples shipped to us from AirProducts (See the first Quarter Report). Consequently, our work has been "labor-intensive" due to the logistics of handling many "unknown" cultures. Yet, in my opinion, we will not be able to propose "alternative ways" to control and/or eliminate the contamination problems until we understand some of the "fundamental cultural and genetic basis" that regulate interactions among organisms.

CONCLUSIONS AND RECOMMENDATIONS

We have not tested ***Gluconacetobacter liquefaciens*** the most important organism that AirProducts personnel have identified as a potential cause (directly or indirectly) of emulsion contamination problems. From discussion, with Dr. Rabasco, we will be receiveing this species among about 30 other isolates. As we modify our approach, we will be able to generate more fundamental information.

I recommend that we continue the **cross-streak interaction experiments**, and **challenge-growth experiments** with *Gluconacetobacter liquefaciens,* and also include the three "producer strains" from my work and proceed with the "known" organisms from AirProducts.

G-32-634
#3

THIRD QUARTER PROGRESS REPORT
(March 1, 2001 - May 31, 2001)

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIONS: BEHAVIOR OF MICROORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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I. EXECUTIVE SUMMARY

Research described in Quarter Reports (# 1 and #2) dealt with studies on **"unknown microorganisms"** in Emulsion Samples that contained: (I) **natural microbial contaminants**, or (ii) Emulsion Samples that were **intentionally "inoculated" with microorganisms by Air Product personnel. We assigned these organisms "codes", because they were "unknown" to us).** During subsequent discussions of results described in those reports with Dr. John Rabasco, other Air Product Personnel, and a Consultant (Dr. Fred Passman), a decision was made to **redirect our research efforts on this project to "FOCUS" on "previously identified organisms"(found in emulsion samples)** that would be shipped to me by Air Product Personnel. I received **twenty-seven cultures** on March 21, 2001. Two of those cultures (*Sphingobacterium spiritorum* and *Cladosporium*) arrived in a "non-viable" state; based on no growth when "initially" subcultured. The remaining 25 cultures were streaked for isolated colonies on Potato Dextrose Agar to determine "purity". Then, each of these organisms was suspended in a separate vial of Mueller-Hinton Broth + 15% glycerol, and stored at -85°C in a Revco Freezer.

Experimental results described in this **Third-Quarter Report** are divided into three categories: **(I) Statistical Analyses of Parameters in Growth Studies.** Objectives were to establish "Experimental Variability" (for the researcher who performed the experiment) **using four "high-priority" organisms (i.e., the most common organisms that have been found in contaminated emulsions):** *Gluconoacetobacter liquifaciens* (GABL), *Methylobacterium*

extorquens, Acinetobacter baumannii, Candida tropicalis, and *Trichoderma viride*. **(ii) Cross-Streak Experiments.** Objectives were to identify isolates that exhibit a dominant role in contaminated emulsions. **(iii) Growth-Interaction Studies.** Objectives were to demonstrate which organisms grew **(i.e., increase in population size), and/or caused physical changes in a single Emulsion Sample (Airflex 400 [before biocide], Lot #C-145).**

Preliminary results from studies completed in each category are summarized below.

Category **(I). The Spread-Plate Method was used to measure growth, and statistical analysis of "three trials" (for each sampling period during growth) were performed to determine "Experimental Variability" using four organism:** *Gluconoacetobacter liquifaciens* (GABL), *Candida tropicalis, Alcaligenes faecalis,* and *Acinetobacter baumannii*. The following statistical values were calculated: the Mean (Average), the Standard Deviation, and the 95% Confidence Interval. This phase of our research involved numerous logistical manipulations, and was extremely time-consuming. By calculating these statistical values, we generated additional data, but the significance of these data may not justify the efforts (in person-hours required to generate them).

Category **(ii). Cross-Streak Interaction Experiments**. We evaluated five organisms for their ability to function as a **"producer-organism" (i.e., center streak on agar plates)**: *Gluconoacetobacter liquifaciens* (GABL), *Acinetobacter baumannii, Candida tropicalis, Pseudomonas aeruginosa,* and *Corynebacterium ammoniagenes*. It is noteworthy that GABL **inhibited all bacterial isolates** tested (i.e., those that grew on Potato Dextrose Agar at room

temperature). GABL **did not inhibit** any yeast or molds that were tested. It is also noteworthy that *Pseudomonas aeruginosa* **inhibited** GABL. The other three organisms (*Acinetobacter baumannii, Candida tropicalis,* and *Corynebacterium ammoniagenes*) **did not inhibit** any of the organisms tested in these experiments.

Category **(iii). Growth-Interaction Studies.** In **"continuing experiments",** we are used the **Spread-Plate Method** to measure growth of "separate organisms" in a single Emulsion Sample (Airflex 400 [Before Biocide], Lot #C-145). The objectives are to determine which organisms grow (i.e., increase in population size) and/or cause **physical changes in the emulsion,** by using several parameters (pH, odor, appearance, and Phase Separation). During these experiments, **one sample/week** from each of six culture will be analyzed for a total period of eight weeks. The six organisms being evaluated are: *Gluconoacetobacter liquifaciens* (GAB|L) , *Candida tropicalis, Alcaligenes faecalis, Acinetobacter baumannii, Aspergillus,* and *Geotrichum candidum.* At the end of three weeks, GABL is the **only organism that shows growth** (i.e., an increased in population size/week). Another interesting observation is an increase in pH values (i.e., decrease in acidity). We also observed some changes in odor, color, and phase separation for GABL.

It is important to remember that **all results reported** in this report are based on **one experiment** per organism in each category. Consequently, these experiments must be repeated before any **definitive interpretations** can be formulated.

II. OBJECTIVES OF EXPERIMENTS IN THIS REPORT

Specific objectives for experiments **(completed or on-going)** during the period covered in this report are stated below under each **Category of Experiments.**

Category (I). Objectives of Statistical Analyses of Parameters in Growth Studies to Determine "Experimental Variability".

(1) To measure growth (i.e., increase in population size) of each organism using the **Spread-Plate Method..**

(2) To remove three separate samples at each time-period (0hr, 4hr, 8hr, etc.), and calculate the following statistical values: the Mean (Average), the Standard Deviation, and the 95% Confidence Interval.

Category (ii). Objectives of Cross-Streak Interaction Experiments.

(1) To perform each experiment with one of the "high-priority" organisms (i.e., most commonly isolated from emulsion samples); beginning with GABL as the **"producer organism".**

(2) To generate date that will demonstrate **inhibition** of other organisms by **"producer organisms"**, on the basis of **substances excreted into the agar medium**.

Category (iii). Objectives of Growth-Interaction Experiments.

(1) To use the **Spread-Plate Method** to measure growth of "separate organisms"; in a single Emulsion Sample (Airflex 400 [Before Biocide], Lot #C-145) for a period of **eight weeks.**

(2) To analyze one sample/week from each organism by measuring growth, and properties of the emulsion (pH, odor, appearance, and phase separation).

III. METHODS/PROCEDURES/PROTOCOLS

I. Protocol for Statistical Calculations **(Appendix I).**

II. Protocol for Growth Measurement using Spread-Plate **(See Page 1, of 1st Quarter Report, dated 12/1/00).**

III. Protocol for the Cross-Streak Interaction Experiment **(See Page 6, of 2nd Quarter Report, dated 3/22/01).**

IV. Protocol for Growth-Interaction Experiment:

a. Use a 500 ml pre-sterilized wide-mouth of a single Emulsion Sample (Airflex 400 [Before biocide], Lot #C-145).

B. Inoculate the Emulsion with 25ml of a "washed" cell suspension (for the designated organism) in Saline (0.85%); standardized by using a spectrophotometer to a concentration of approximately 1 X 10^6 CFU/ml.

C. Incubate each inoculated sample at Room temperature (26° C- 28 °C) for eight weeks. Record data at time periods: 0hr, 1Wk, 2Wk, 3Wk, 4Wk, and 8Wk.

(1) Prior to opening the sample (at each time period), record physical Observations: Odor, Appearance, and Phase Separation (See Protocol, Appendix II).

(2) Use a sterile syringe to remove a 30 ml-sample for growth Measurement using the spread-plate method, and use the remainder of the sample for pH measurements (using Rabasco's Procedure sent to me).

IV. RESULTS

Results from experiments described in this report are discussed in separate categories.

Category **(I). Results from Statistical Analysis and Values to Determine "Experimental Variability".** Four organisms was analyzed in this phase of the research. Growth curve data and statistical values (the mean, standard deviation, and 95% confidence level) are shown for each test organism: *Gluconoacetobacter liquifaciens* **(Table 1, and Figure a)**, *Candida tropicalis* **(Table 2, and Figure B)**, *Alcaligenes faecalis* **(Table 3, and Figure C)**, and *Acinetobacter baumannii* **(Table 4, and Figure D)**.

Catergory **(ii). Results from Cross-Streak Interaction Experiments.** We tested five organisms for their ability to function as a **"producer organism" (i.e., center streak on an agar plate)**, and secrete substances into the medium that may subsequently inhibit the growth of other organisms; inoculated on the same plate in cross-streaks perpendicular to the "center streak". The results obtained for *Gluconoacetobacter liquifaciens* (GABL) are shown in **Table 5, and Figures 1 - 6).** GABL inhibited all bacterial organisms tested, and **did not inhibit** any of the yeasts or molds. It is interesting to note that *Pseudomonas aeruginosa* **inhibited GABL**, and these data are shown in **Table 8, and Figures 22 - 29).** The other three organisms **did not inhibit** any of the cross-streak organisms used in these experiments. These data are shown for each organism in the following Tables and Figures: *Acinetobacter baumannii* **(Table 6, and Figures 7 - 13)**, *Candida tropicalis* **(Table 7, and Figures 14 - 21)**, and *Corynebacterium ammoniagenes* **(Table 9, and Figures 30 - 36).**

Category (iii). Results from Growth-Interaction Studies. These experiments are **"on-going"**, and will continue for a period of eight weeks. Consequently, data shown for each organism covers only a **few weeks; and no "definitive interpretations"** will be presented. Six organisms are being evaluated in a single emulsion type (Airflex 400 [Before Biocide], Lot #C-145). The results from growth determinations, pH, and other physical changes were recorded at each sampling "time period". **Qualitative Observations for certain physical changes** (odor, appearance, and appearance) were scored as described in **Appendix II.** Data obtained (at the close of this reporting period) are shown for each of six organisms. *Gluconoacetobacter liquifaciens* **(Table 10)** is the **only organism that shows growth** (i.e., an increase in population size/week). Other changes for this organism are: pH values (i.e., decrease in acidity), changes in odor, appearance, and phase separation. No changes were noted for each of the other organisms: *Candica tropicalis* **(Table 11),** *Alcaligenes faecalis* **(Table 12),** *Acinetobacter baumannii* **(Table 13),** *Aspergillus* **(Table 14),** and *Geotrichum candidum* **(Table 15).**

V. INTERPRETATIONS AND SIGNIFICANCE

From observations of experiments performed in **Category (I),** designed to determine **experimental variability**, we generated additional data. However, this phase of our research involved numerous logistical manipulations, and was extremely time-consuming. The significance of these data may not justify the efforts (in person-hours required to generate them). Consequently, I recommend that we **discontinue these statistical analyses.**

Results obtained from **Cross-Streak Interaction Experiments in Category (ii)** show that GABL **inhibited all bacterial cultures tested.** However, GABL **did not inhibit any**

Yeasts or Molds. It is also noteworthy that *Pseudomonas aeruginosa* inhibited GABL.

In **Category (iii) Growth-Interaction Studies**, GABL is the only organisms tested that shows growth, and some changes in physical parameters in Airflex 400 [Before Biocide), Lot# C-145. This experiment is still in progress, and we will continue to collect data until the end of the eight-week period.

VI. CONCLUSIONS AND RECOMMENDATIONS

We do not know the significance of these results, because **these experiments have only been performed one time, and must be "repeated".** However, these preliminary findings suggest that we should **continue "cross-streak" experiments using GABL against the remainder of organisms shipped to us by Air Products' personnel; identification of these organisms are shown in Appendix III.**

During the 4th Quarter of this Grant, we recommend **the following experiments:**

1) Repeat the Cross-Streak Experiment (GABL as "center streak"), against the same organisms.

2) Repeat the reciprocal Cross-Streak Experiments; using GABL vs. *Pseudomonas aeruginosa.*

3) Repeat the eight-week "Growth-Interaction Experiments" using Airflex 400 [Before Biocide], Lot #C-145, inoculated with GABL: a) measure growth, pH, odor, appearance, and phase separation as describe in this report, and b) **in addition**, a Chemist will analyze the Emulsion for the following: pH & conductivity, viscosity, grit, molecular weight (aqueous phase & solid phase), and particle size distribution. **(Note: At this time, we have not located the specific Chemist on Tech's Campus, but hopefully the individual will be located in time to perform these tests).**

Table 1: **Standard growth curve of *Gluconoacetobacter liquefaciens***

Date/Time	Trial No.	%T	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Viable Counts Average cfu/ml
4/17/01	No. 1		TN	TN	TN	184	TF	TF	
0hr	No. 2	90%	TN	TN	TN	205	TF	TF	$2.15 * 10^7$
	No. 3		TN	TN	TN	258	TF	TF	
4/17/01	No. 1		TN	TN	TN	272	TF	TF	
4hr	No. 2	90%	TN	TN	TN	290	TF	TF	$2.76 * 10^7$
	No. 3		TN	TN	TN	268	TF	TF	
4/17/01	No. 1		TN	TN	TN	267	TF	TF	
8hr	No. 2	89%	TN	TN	TN	238	TF	TF	$2.47 * 10^7$
	No. 3		TN	TN	TN	238	TF	TF	
4/18/01	No. 1		TN	TN	TN	184	TF	TF	
24hr	No. 2	89%	TN	TN	TN	110	TF	TF	$1.52 * 10^7$
	No. 3		TN	TN	TN	163	TF	TF	
4/19/01	No. 1		TN	TN	TN	295	TF	TF	
48 hr	No. 2	80%	TN	TN	TN	298	TF	TF	$2.96 * 10^7$
	No. 3		TN	TN	TN	297	TF	TF	
4/20/01	No. 1		TN	TN	TN	TN	39	TF	
72 hr	No. 2	64%	TN	TN	TN	TN	38	TF	$3.63 * 10^7$
	No. 3		TN	TN	TN	TN	32	TF	

TN = Too numerous (> 300), TF = Too few (< 30)

Descriptive Statistics for Gluconoacetobacter liquefaciens (10-5 concentrations) at 0hr, 4hr, 8hr, 24hr, 48hr, 72hr

Variable	N	Mean	Median	TrMean	StDev	SE Mean
0hr	3	215.7	205.0	215.7	46.1	22.0
4hr	3	276.67	272.00	276.67	11.72	6.77
8hr	3	247.67	238.00	247.67	16.74	9.67
24hr	3	147.3	163.0	147.3	99.1	32.0
48hr	3	296.67	297.00	296.67	1.53	1.86
72hr	3	353.3	350.0	353.3	21.9	21.9

Variable	Minimum	Maximum	Q1	Q3
0hr	184.0	258.0	184.0	258.0
4hr	268.00	290.00	268.00	290.00
8hr	238.00	267.00	238.00	267.00
24hr	110.0	184.0	110.0	184.0
48hr	295.00	298.00	295.00	298.00
72hr	320.0	390.0	320.0	390.0

Figure A.

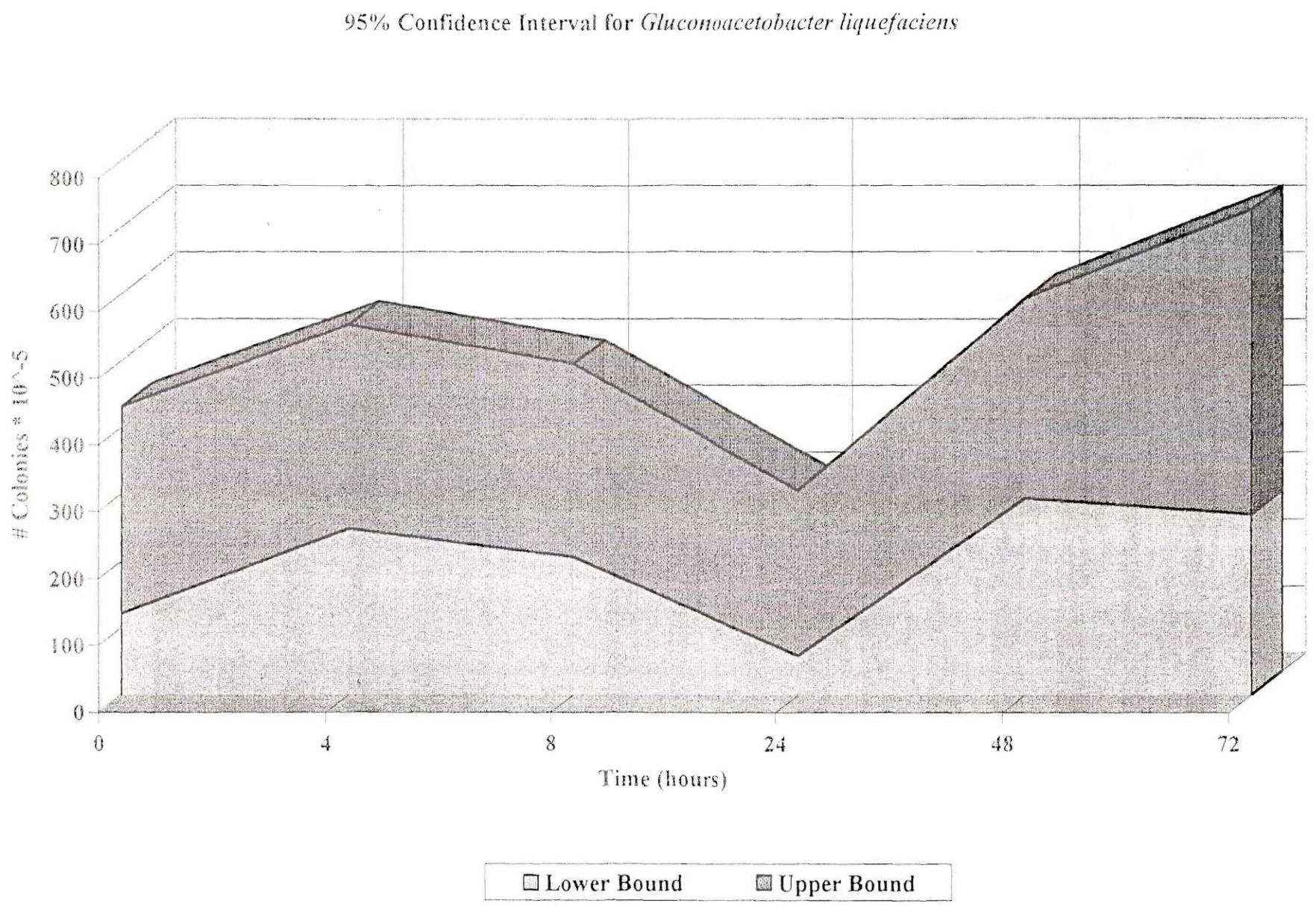
95% Confidence Interval for *Gluconoacetobacter liquefaciens*
Colonies * 10^-5
800
700
600
500
400
300
200
100
0
0
4
8
24
48
72
Time (hours)
Lower Bound
Upper Bound

Table 2: **Standard growth curve of *Candida tropicalis***

Date/Time	Trial No.	%T	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Viable Counts Average cfu/ml
4/23/01	No. 1		TN	233	TF	TF	TF	0	
0hr	No. 2	90%	TN	296	35	TF	0	0	$2.61 * 10^5$
	No. 3		TN	255	31	TF	0	0	
4/23/01	No. 1		-	TN	41	TF	0	0	
4hr	No. 2	86%	-	TN	49	TF	TF	0	$4.4 * 10^5$
	No. 3		-	TN	42	TF	0	0	
4/23/01	No. 1		-	TN	113	TF	0	0	
8hr	No. 2	70%	-	TN	82	TF	TF	0	$9.8 * 10^5$
	No. 3		-	TN	101	TF	0	0	
4/24/01	No. 1		-	TN	TN	52	0	0	
24hr	No. 2	19%	-	TN	TN	50	TF	0	$4.4 * 10^6$
	No. 3		-	TN	TN	30	TF	0	

TN = Too numerous (>300), TF = Too few (< 30)

Descriptive Statistics Candida Tropicalis (10-4 Concentration) at 0hr, 4hr, 8hr, 24hr

Variable	N	Mean	Median	TrMean	StDev	SE Mean
0hr	3	29.77	31.00	29.77	5.95	3.43
4hr	3	44.00	42.00	44.00	4.36	2.52
8hr	3	98.67	101.00	98.67	15.63	9.02
24hr	3	440.0	500.0	440.0	121.7	70.2

Variable	Minimum	Maximum	Q1	Q3
0hr	23.30	35.00	23.30	35.00
4hr	41.00	49.00	41.00	49.00
8hr	82.00	113.00	82.00	113.00
24hr	300.0	520.0	300.0	520.0

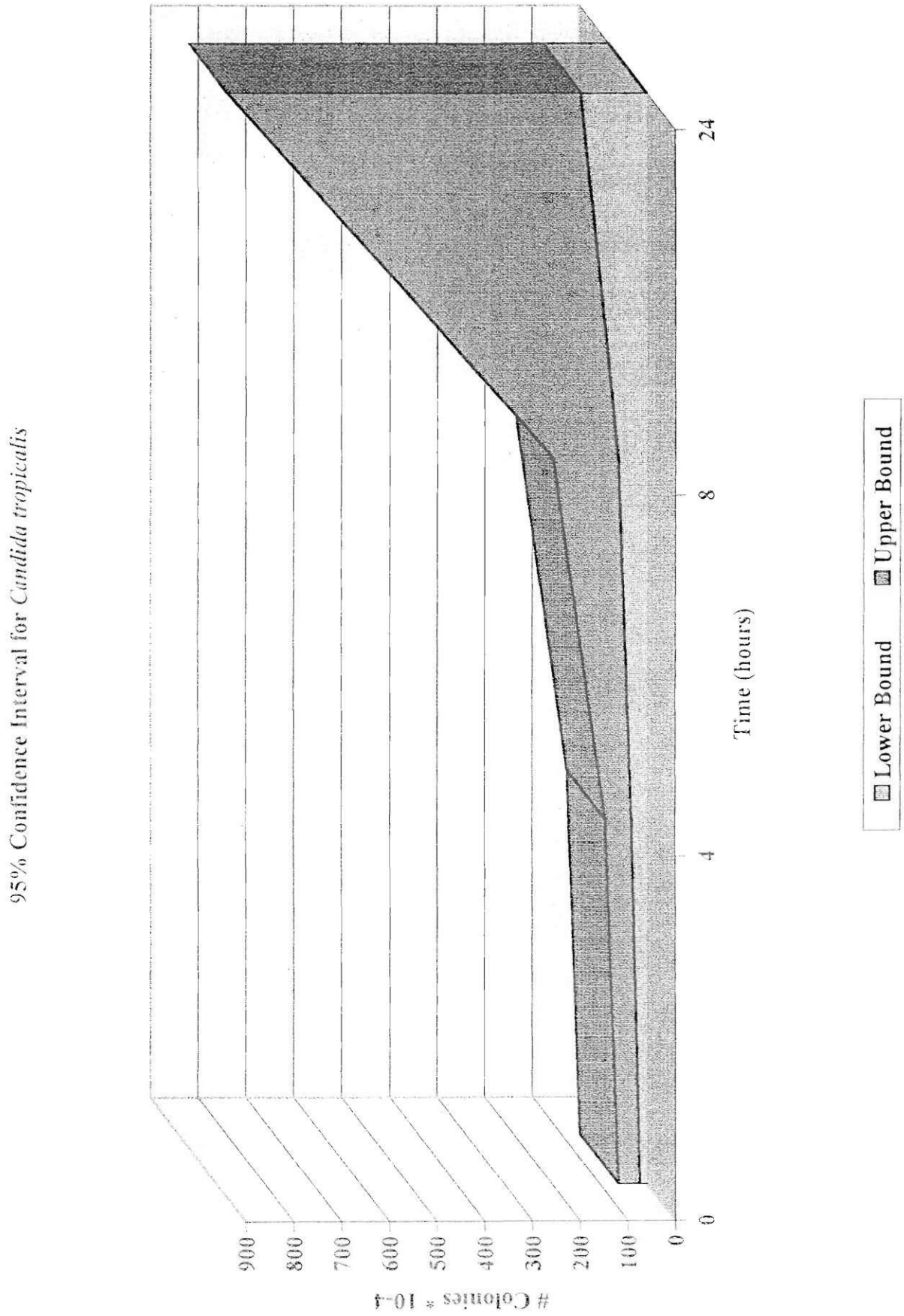
95% Confidence Interval for Candida tropicalis
Colonies * 10-4
900
800
700
600
500
400
300
200
100
0
0
4
8
24
Time (hours)
Lower Bound
Upper Bound

Figure B.

Table 3. **Standard growth curve of *Alcaligenes faecalis***

Date/Time	Trial No.	%T	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Viable Counts Average cfu/ml
5/2/01	No. 1		TN	TN	TN	230	TF	TF	
0hr	No. 2	90%	TN	TN	TN	190	TF	TF	$2.03 * 10^7$
	No. 3		TN	TN	TN	190	TF	TF	
5/2/01	No. 1		-	TN	TN	200	TF	TF	
4hr	No. 2	50%	-	TN	TN	186	TF	TF	$2.1 * 10^7$
	No. 3		-	TN	TN	246	TF	TF	
5/2/01	No. 1		-	TN	TN	191	TF	TF	
8hr	No. 2	45%	-	TN	TN	219	TF	TF	$2.21 * 10^7$
	No. 3		-	TN	TN	253	TF	TF	
5/3/01	No. 1		-	TN	TN	TN	62	TF	
24hr	No. 2	25%	-	TN	TN	TN	89	TF	$7.5 * 10^7$
	No. 3		-	TN	TN	TN	74	TF	

TN = Too numerous (>300), TF = Too few (< 30)

Descriptive Statistics for Alcaligenes faecalis at Concentration of 10-6 at 0hr, 4hr, 8hr , 24hr

Variable	N	Mean	Median	TrMean	StDev	SE Mean
0hr	3	38.67	39.00	38.67	8.50	4.91
4hr	2	284.00	284.00	284.00	2.83	2.00
8hr	3	316.67	320.00	316.67	15.28	8.82
24hr	3	460.0	470.0	460.0	26.5	15.3

Variable	Minimum	Maximum	Q1	Q3
0hr	30.00	47.00	30.00	47.00
4hr	282.00	286.00	*	*
8hr	300.00	330.00	300.00	330.00
24hr	430.0	480.0	430.0	480.0

*only two sets of data were available, thus unable to be calculated

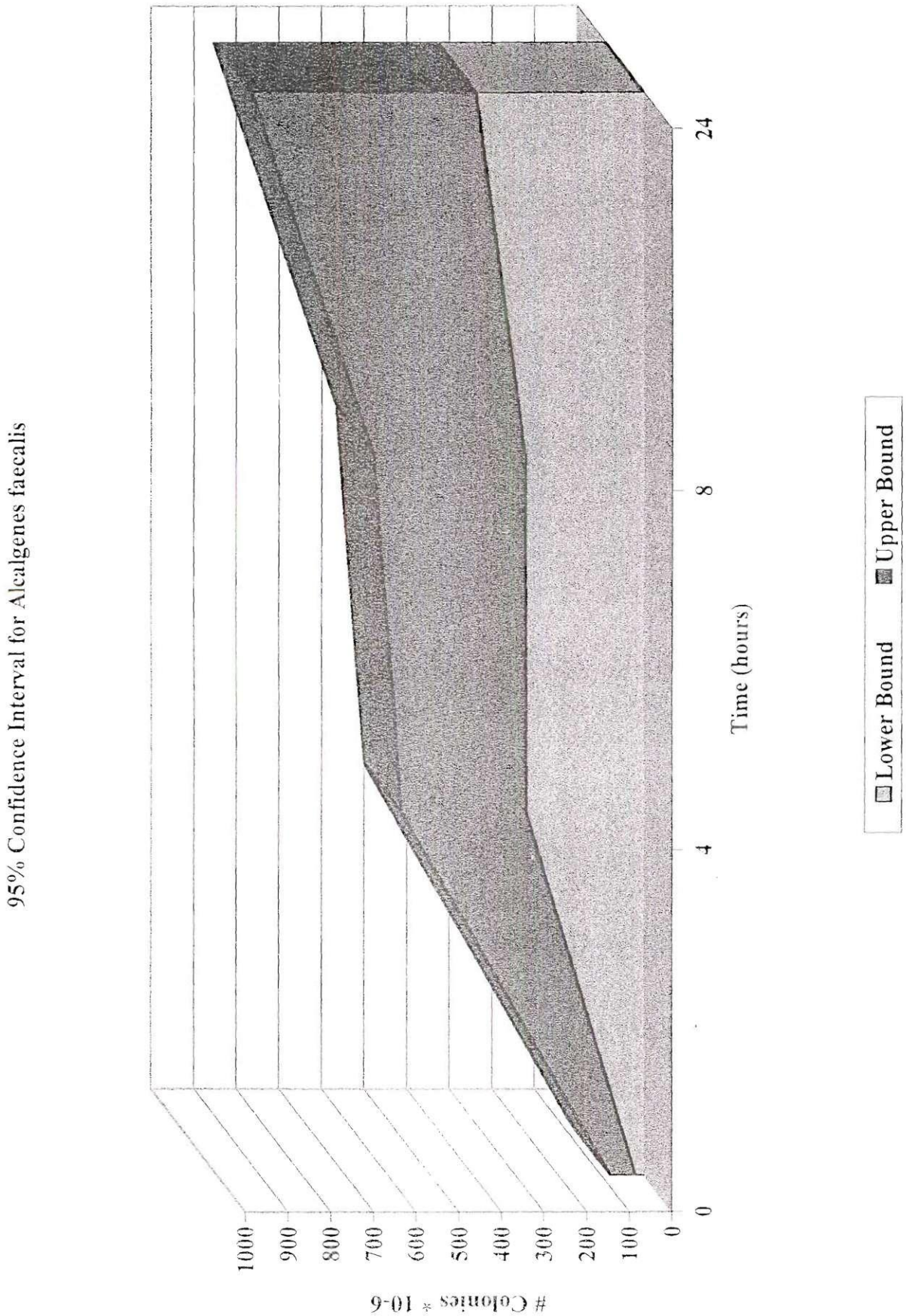
95% Confidence Interval for Alcalgenes faecalis
Colonies * 10-6
1000
900
800
700
600
500
400
300
200
100
0
0
4
8
24
Time (hours)
Lower Bound
Upper Bound

Figure C.

Table 4: **Standard growth curve of *Acinetobacter baumannii***

Date/Time	Trial No.	%T	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Viable Counts Average cfu/ml
5/1/01	No. 1		TN	TN	TN	230	TF	TF	
0hr	No. 2	90%	TN	TN	TN	190	TF	TF	$2.03 * 10^7$
	No. 3		TN	TN	TN	190	TF	TF	
5/1/01	No. 1		-	TN	TN	200	TF	TF	
4hr	No. 2	86%	-	TN	TN	186	TF	TF	$2.1 * 10^7$
	No. 3		-	TN	TN	246	TF	TF	
5/1/01	No. 1		-	TN	TN	191	TF	TF	
8hr	No. 2	80%	-	TN	TN	219	TF	TF	$2.21 * 10^7$
	No. 3		-	TN	TN	253	TF	TF	
5/2/01	No. 1		-	TN	TN	TN	62	TF	
24hr	No. 2	65%	-	TN	TN	TN	89	TF	$7.5 * 10^7$
	No. 3		-	TN	TN	TN	74	TF	

TN = Too numerous (>300), TF = Too few (< 30)

Descriptive Statistics for Acinetobacter baumannii (Concentrations of 10-5) at 0hr, 4hr, 8hr, 24hr

Variable	N	Mean	Median	TrMean	StDev	SE Mean
0hr	3	203.3	190.0	203.3	23.1	13.3
4hr	3	210.7	200.0	210.7	31.4	18.1
8hr	3	221.0	219.0	221.0	31.0	17.9
24hr	3	750.0	740.0	750.0	135.3	78.1

Variable	Minimum	Maximum	Q1	Q3
0hr	190.0	230.0	190.0	230.0
4hr	186.0	246.0	186.0	246.0
8hr	191.0	253.0	191.0	253.0
24hr	620.0	890.0	620.0	890.0

Figure D.

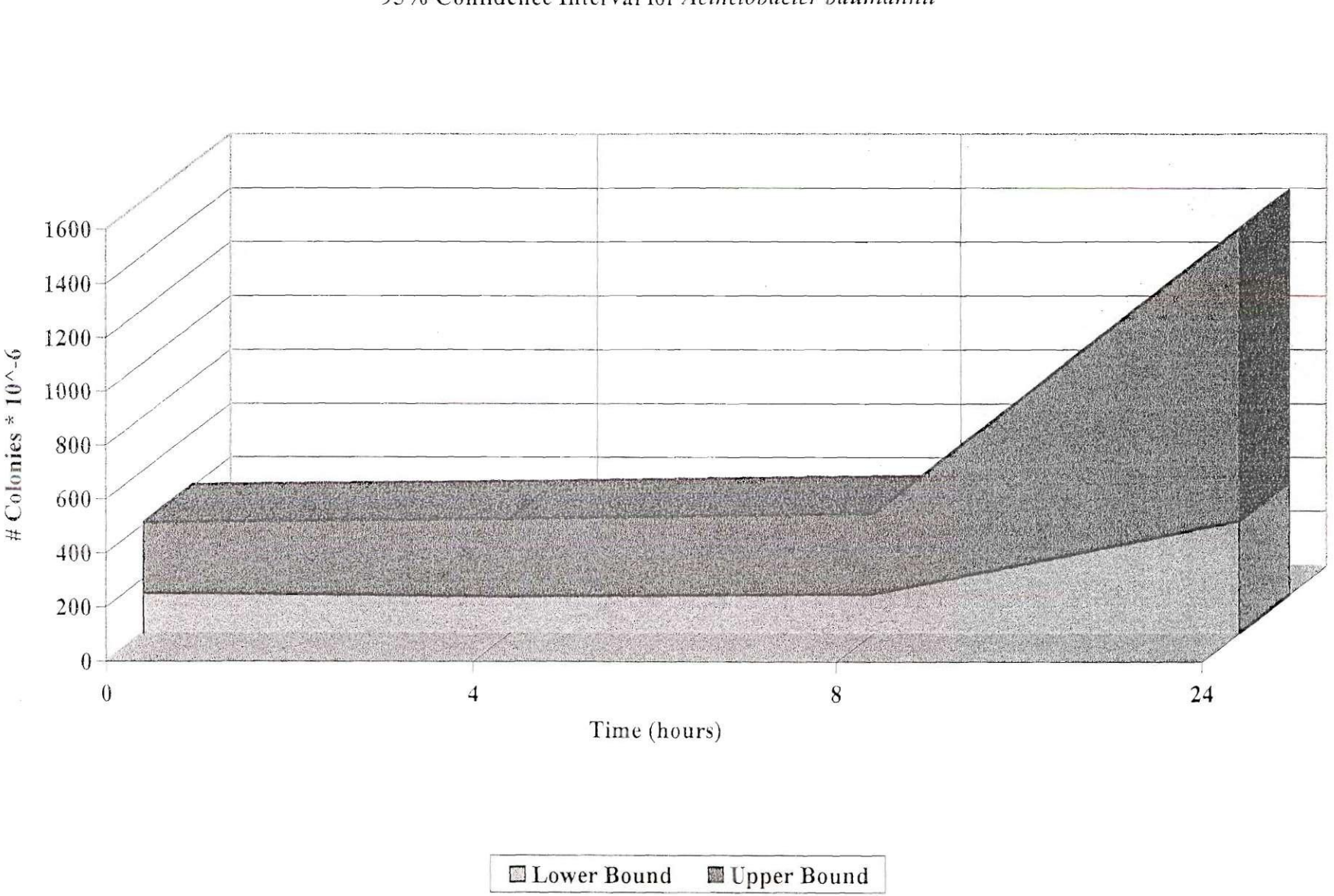
95% Confidence Interval for *Acinetobacter baumannii*
Colonies * 10^-6
1600
1400
1200
1000
800
600
400
200
0
0
4
8
24
Time (hours)
Lower Bound
Upper Bound

Table 5 **Results for *Gluconoacetobacter liquefaciens* Cross-Streak Interactions**

Cross-Streak Organism	Media	Incubation for 24hr at 37°C or otherwise indicated	Inhibition (+) or No inhibition (-)
Alcaligenes faecalis	Potato Dextrose Agar	+	+
Pseudomonas stutzer	Does not grow on potato dextrose agar		
Providencia rettgeri	Potato Dextrose Agar	+	+
Klebsiella pneumoniae	Potato Dextrose Agar	+	+
Rhodoturula glutinis	Potato Dextrose Agar	72 hr / room temp	-
Geotrichum candidum	Potato Dextrose Agar	+	-
Candida tropicalis	Potato Dextrose Agar	+	-
Pseudomonas aeruginosa	Potato Dextrose Agar	+	+
Candida guillermondi	Potato Dextrose Agar	+	-
Sphingobacterium spiritivorum	Potato Dextrose Agar	48 hr / 37°C	+
Burkholderia gepacia	Potato Dextrose Agar	+	+
Proteus vulgaris	Potato Dextrose Agar	72 hr / room temp	+
Enterobacter aerogenes	Potato Dextrose Agar	+	+
Methylobacterium extorquens	Potato Dextrose Agar	No growth	
Trichoderma viride	Potato Dextrose Agar	48 hr / 37°C	-
Shewanella putrefaciens	Potato Dextrose Agar	+	+
Chryzeobacterium meningosepticum	Potato Dextrose Agar	48 hr / 37°C	+
Aspergillus	Potato Dextrose Agar	48 hr / 37°C	-
Ralstonia pickettii	Does not grow on potato dextrose agar		
Sporothrix	Potato Dextrose Agar	72 hr / room temp	-
Serratia liquefaciens	Potato Dextrose Agar	+	+
Escherichia coli	Potato Dextrose Agar	+	+
Aeromonas hydrophilia	Potato Dextrose Agar	+	+
Acinetobacter baumannii	Potato Dextrose Agar	+	+
Corynebacterium ammoniagenes	Does not grow on potato dextrose agar		

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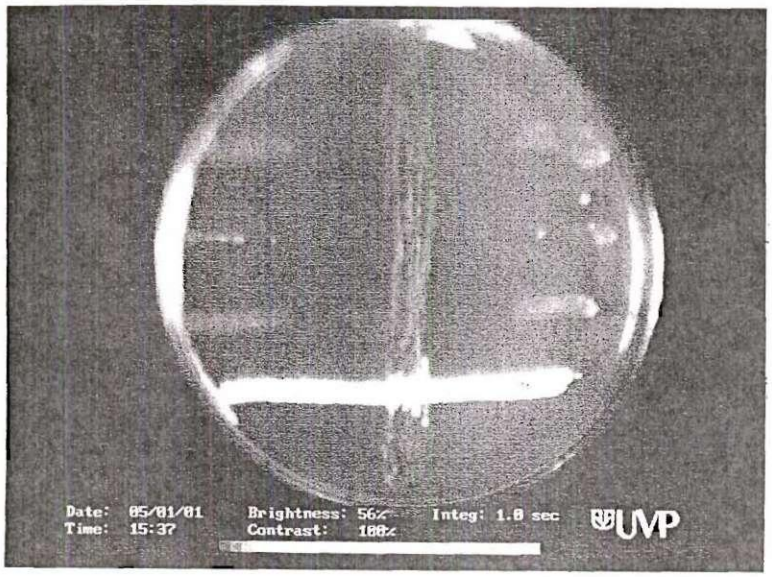

FIG. 1. **A.)** Center-Streak Organism: *Gluconoacetobacter liquefaciens*. Cross-Streak Organisms: **1.)** *Alcaligenes faecalis* (+) **2.)** *Providencia rettgeri* (+) **3.)** *Klebsiella pneumoniae* (+) **4.)** *Candida tropicalis* (-) Growth inhibition (+), No growth inhibition (-)

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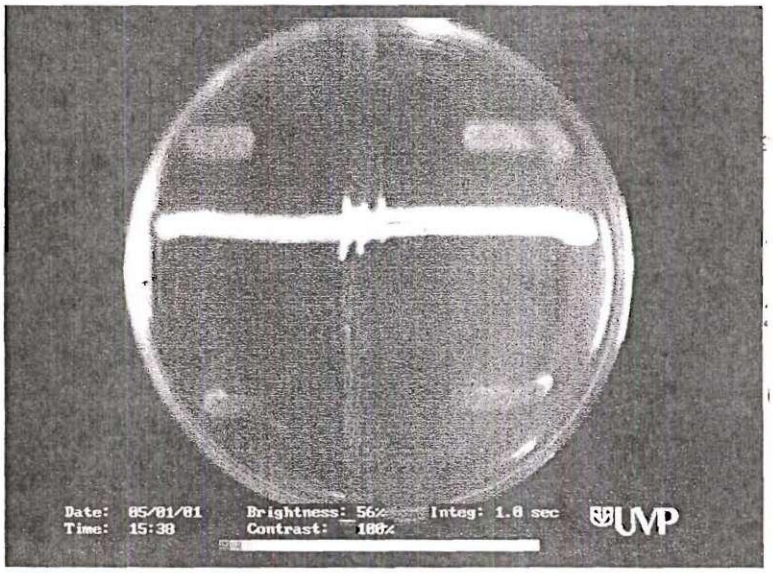

FIG. 2. **A.)** Center-Streak Organism: *Gluconoacetobacter liquefaciens*. Cross-Streak Organisms: **1.)** *Pseudomonas aeruginosa* (+) **2.)** *Candida guillermondi* (-) **3.)** *Burkholderia gepacia*(+) **4.)** *Enterobacter aerogenes* (+) Growth inhibition (+), No growth inhibition (-).

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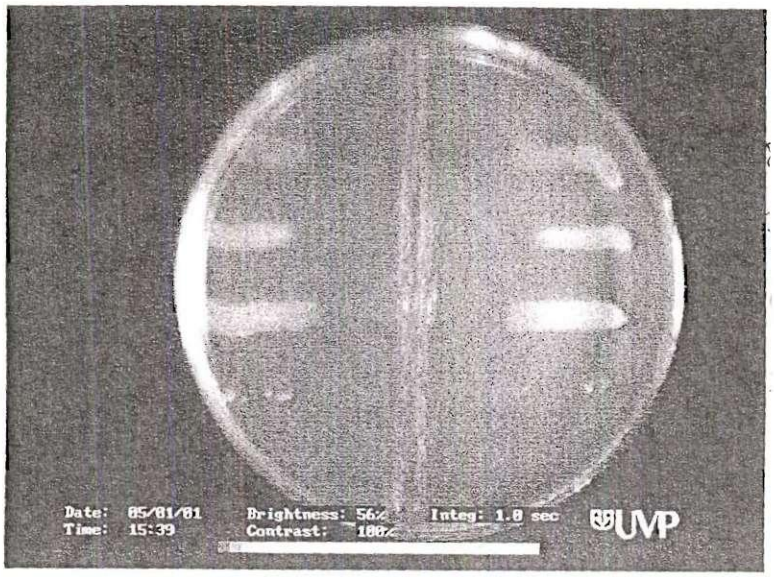

FIG. 3. **A.)** Center-Streak Organism: *Gluconoacetobacter liquefaciens*. Cross-Streak Organisms: **1.)** *Shewanella putrefaciens* (+) **2.)** *Serratia liquefaciens (+)* **3.)** *Escherichia coli* (+) **4.)** *Aeromonas hydrophilia* (+) Growth inhibition (+), No growth inhibition (-).

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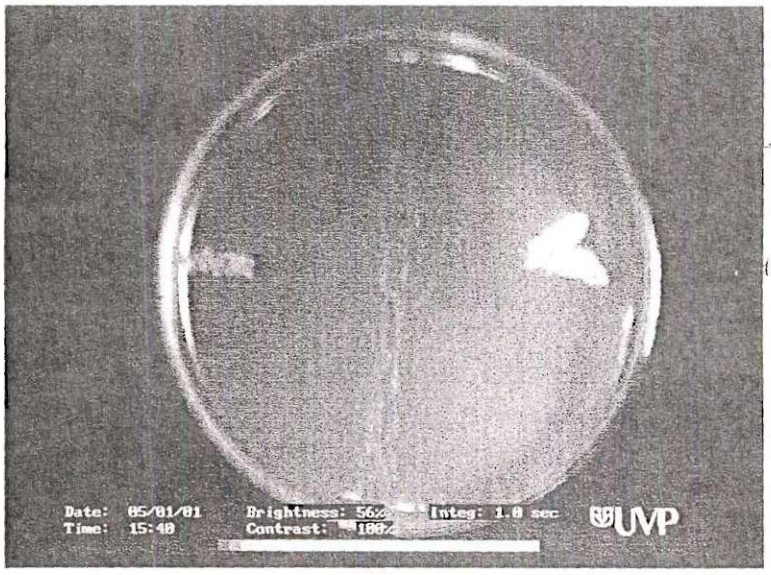

FIG. 4. **A.)** Center-Streak Organism: *Gluconoacetobacter liquefaciens*.. Cross-Streak Organisms: **1.)** *Acinetobacter baumannii* (+) Growth inhibition (+), No growth inhibition (-).

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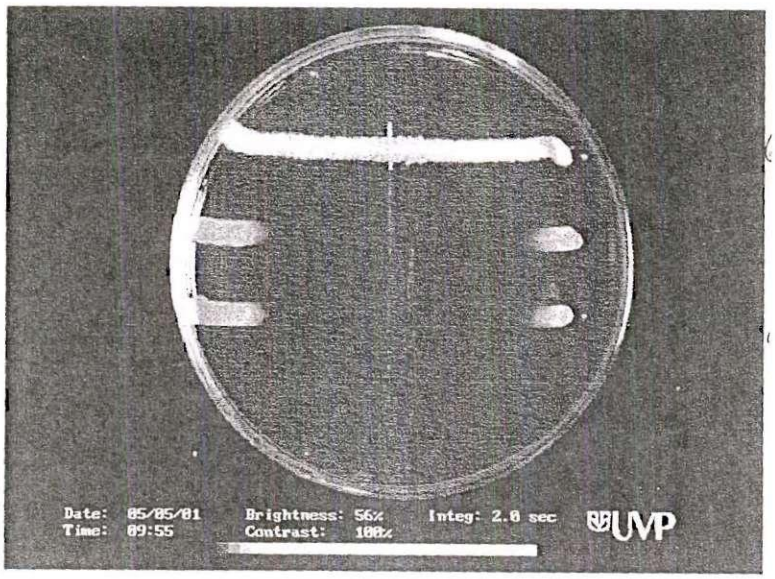

FIG. 5. A.) Center-Streak Organism: *Gluconoacetobacter liquefaciens*. Cross-Streak Organisms: 1.) *Rhodoturula glutinis* (-) 2.) *Sphingobacterium spiritivorum* (+) 3.) *Chryzeobacterium meningosepticum* (+) Growth inhibition (+), No growth inhibition (-)

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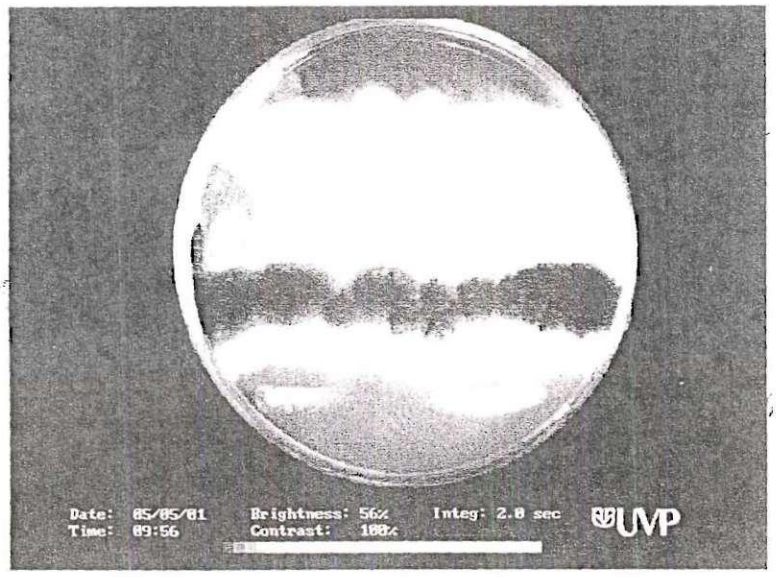

FIG. 6. A.) Center-Streak Organism: *Gluconoacetobacter liquefaciens*. Cross-Streak Organisms: 1.) *Geotrichum candidum* (-) 2.) *Trichoderma viride* (-) 3.) *Aspergillus* (-) 4.) *Sporothrix* (+) Growth inhibition (+), No growth inhibition (-).

Table 6 **Results for *Acinetobacter baumannii* Cross-Streak Interactions**

Cross-Streak Organism	Media	Incubation for 24hr at 37°C or otherwise indicated	Inhibition (+) or No inhibition (-)
Alcaligenes faecalis	Trypticase Soy Agar	+	-
Pseudomonas stutzer	Trypticase Soy Agar	+	-
Providencia rettgeri	Trypticase Soy Agar	+	-
Gluconoacetobacter liquefaciens	Potato Dextrose Agar	48 hr / room temp	-
Klebsiella pneumoniae	Trypticase Soy Agar	+	-
Rhodoturula glutinis	Trypticase Soy Agar	No growth	-
Geotrichum candidum	Trypticase Soy Agar	+	-
Candida tropicalis	Trypticase Soy Agar	+	-
Pseudomonas aeruginosa	Trypticase Soy Agar	+	-
Candida guillermondi	Trypticase Soy Agar	+	-
Sphingobacterium spirivittivorum	Trypticase Soy Agar	48 hr / 37°C	-
Burkholderia gepacia	Trypticase Soy Agar	+	-
Proteus vulgaris	Trypticase Soy Agar	+	-
Enterobacter aerogenes	Trypticase Soy Agar	+	-
Methylobacterium extorquens	Trypticase Soy Agar	6 days / room temp	-
Trichoderma viride	Trypticase Soy Agar	48 hr / 37°C	-
Shewanella putrefaciens	Trypticase Soy Agar	+	-
Chryseobacterium meningosepticum	Trypticase Soy Agar	48 hr / 37°C	-
Aspergillus	Trypticase Soy Agar	48 hr / 37°C	-
Ralstonia pickettii	Trypticase Soy Agar	+	-
Sporothrix	Trypticase Soy Agar	72 hr / room temp	-
Serratia liquefaciens	Trypticase Soy Agar	+	-
Escherichia coli	Trypticase Soy Agar	+	-
Aeromonas hydrophilia	Trypticase Soy Agar	+	-
Corynebacterium ammoniagenes	Trypticase Soy Agar	+	-

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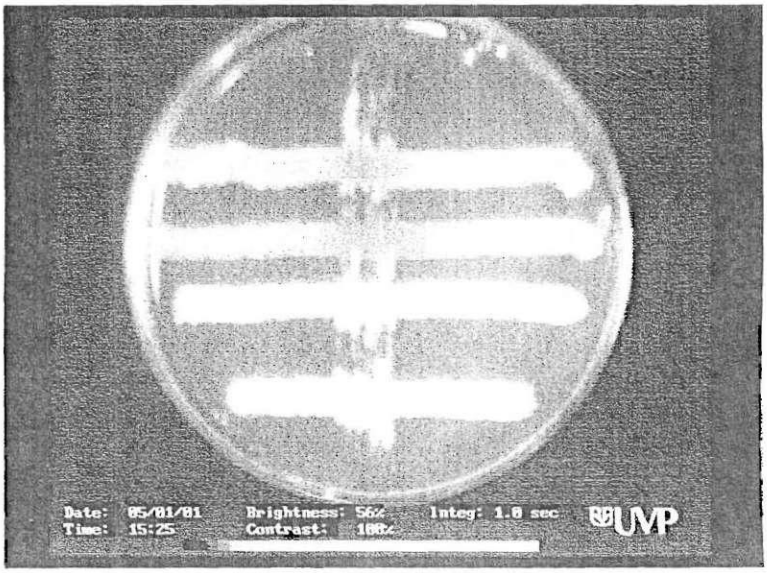

FIG. 7. A.) Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Alcaligenes faecalis* (-) **2.)** *Pseudomonas stutzer* (-) **3.)** *Providencia rettgeri* (-) **4.)** *Klebsiella pneumoniae*(-) Growth inhibition (+), No growth inhibition (-).

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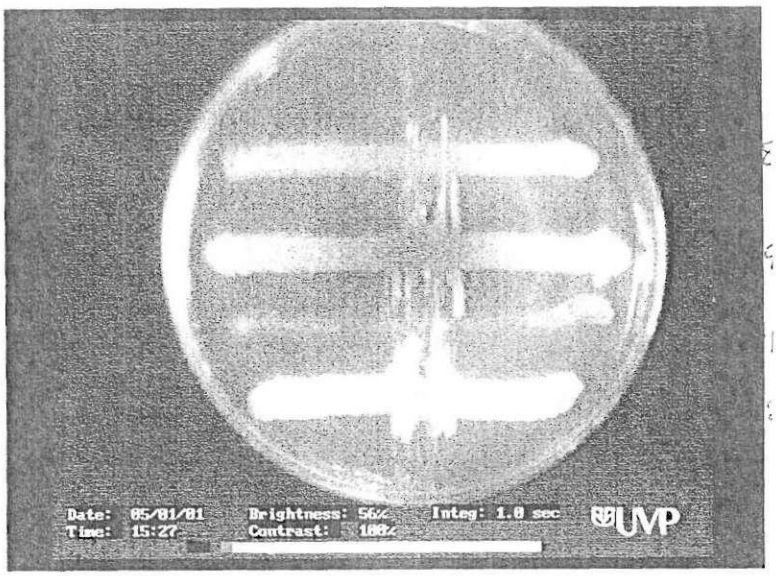

FIG. 8. A.) Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Candida tropicalis* (-) **2.)** *Pseudomonas aeruginosa* (-) **3.)** *Candida guillermondi* (-) **4.)** *Burkholderia gepacia* (-) Growth inhibition (+), No growth inhibition (-).

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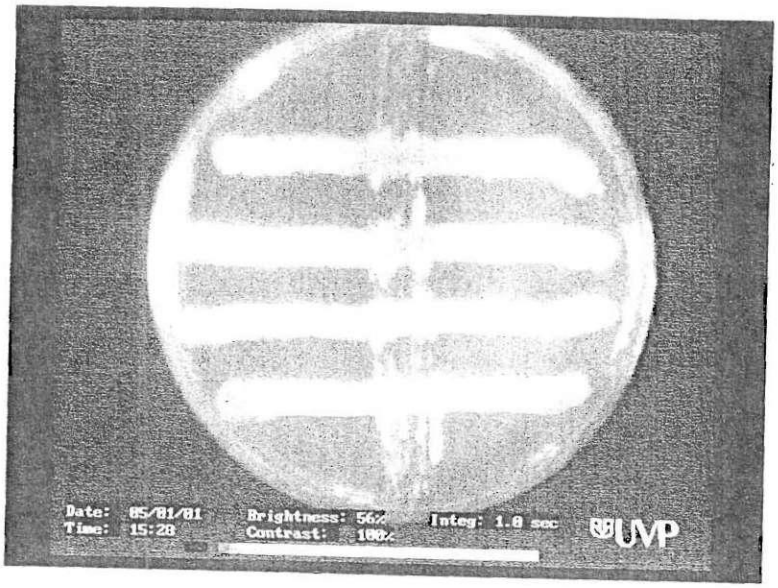

FIG. 9. A.) Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Proteus vulgaris* (-) **2.)** *Enterobacter aerogenes* (-) **3.)** *Shewanella putrefaciens* (-) **4.)** *Ralstonia pickettii* (-). Growth inhibition (+), No growth inhibition (-).

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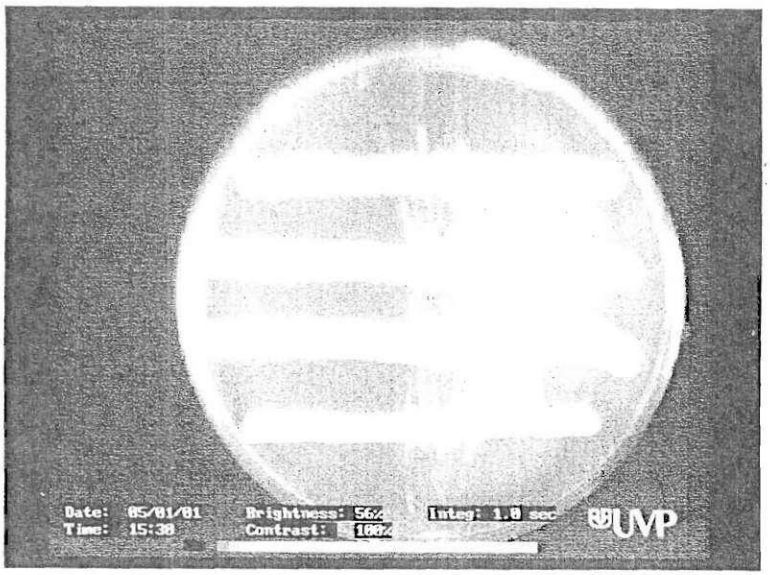

FIG. 10. A.) Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Serratia liquefaciens* (-) **2.)** *Escherichia coli* (-) **3.)** *Aeromonas hydrophilia* (-) **4.)** *Corynebacterium ammoniagenes* (-) Growth inhibition (+), No growth inhibition (-).

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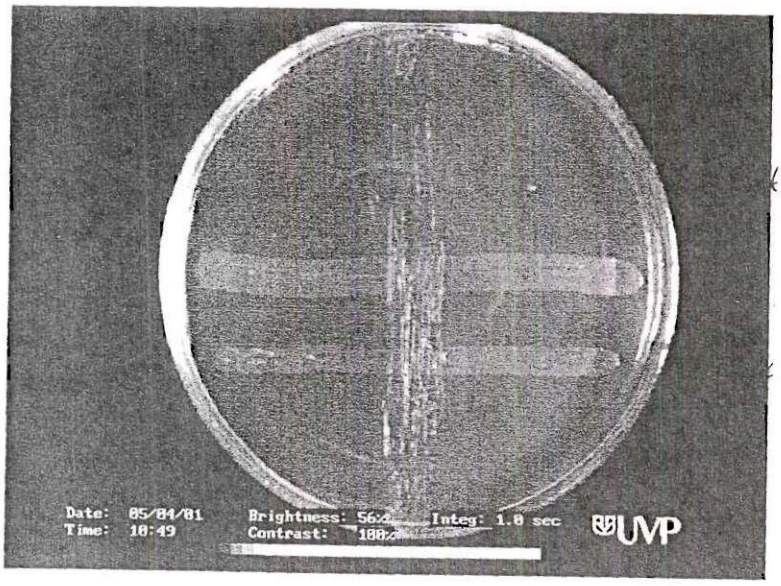

FIG. 10. A.) Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Rhodoturula glutinis* (no growth) **2.)** *Sphingobacterium spiritivorum* (-) **3.)** *Chryzeobacterium meningosepticum* (-) Growth inhibition (+), No growth inhibition (-)

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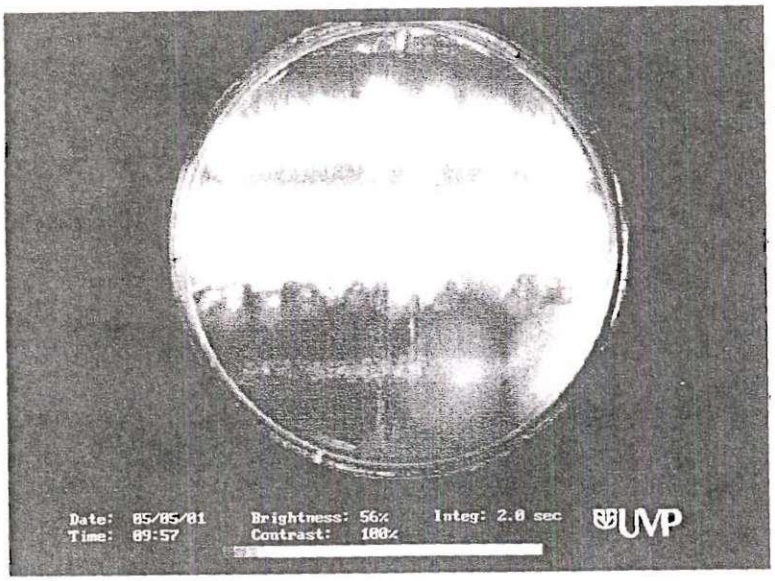

FIG. 11. A.) Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Geotrichum candidum* (-) **2.)** *Trichoderma viride* (-) **3.)** *Aspergillus* (-) **4.)** *Sporothrix* (-) Growth inhibition (+), No growth inhibition (-).

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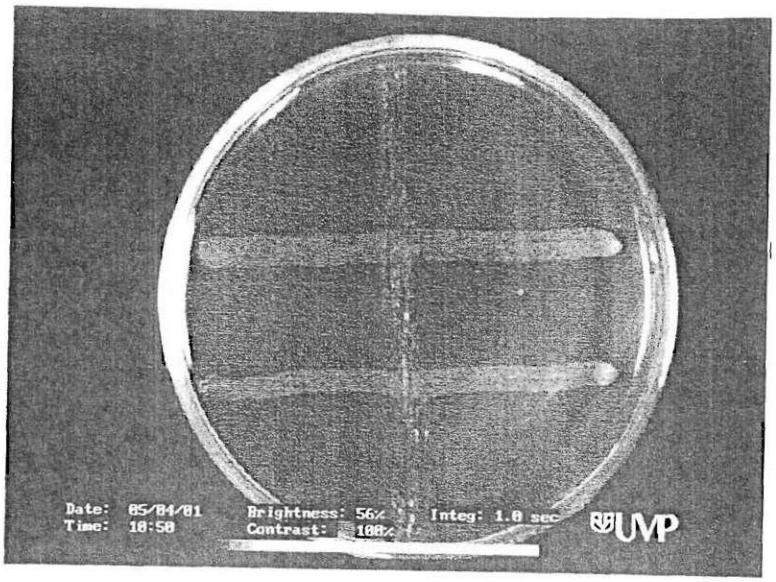

FIG. 12. **A.)** Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Gluconoacetobacter liquefaciens* (-) **2.)** *Gluconoacetobacter liquefaciens* (-) Growth inhibition (+), No growth inhibition (-).

A

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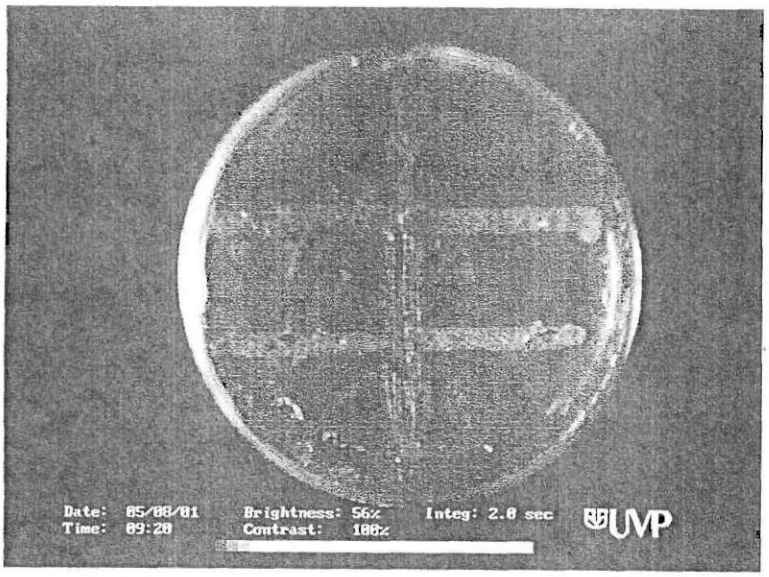

FIG. 13. **A.)** Center-Streak Organism: *Acinetobacter baumannii*. Cross-Streak Organisms: **1.)** *Methylobacterium extorquens* (-) **2.)** *Methylobacterium extorquens* (-) Growth inhibition (+), No growth inhibition (-).

Table 7 **Results for *Candida tropicalis* Cross-Streak Interactions**

Cross-Streak Organism	Media	Incubation for 24hr at 37°C or otherwise indicated	Inhibition (+) or No inhibition (-)
Alcaligenes faecalis	Trypticase Soy Agar	+	-
Pseudomonas stutzer	Trypticase Soy Agar	+	-
Providencia rettgeri	Trypticase Soy Agar	+	-
Gluconoacetobacter liquefaciens	Potato Dextrose Agar	48 hr / room temp	-
Klebsiella pneumoniae	Trypticase Soy Agar	+	-
Rhodoturula glutinis	Trypticase Soy Agar	No growth	-
Geotrichum candidum	Trypticase Soy Agar	+	-
Pseudomonas aeruginosa	Trypticase Soy Agar	+	-
Candida guillermondi	Trypticase Soy Agar	+	-
Sphingobacterium spirivitivorum	Trypticase Soy Agar	48 hr / 37°C	-
Burkholderia gepacia	Trypticase Soy Agar	+	-
Proteus vulgaris	Trypticase Soy Agar	+	-
Enterobacter aerogenes	Trypticase Soy Agar	+	-
Methylobacterium exterquens	Trypticase Soy Agar	6 days / room temp	-
Trichoderma viride	Trypticase Soy Agar	48 hr / 37°C	-
Shewanella putrefaciens	Trypticase Soy Agar	+	-
Chryzeobacterium meningosepticum	Trypticase Soy Agar	48 hr / 37°C	-
Aspergillus	Trypticase Soy Agar	48 hr / 37°C	-
Ralstonia pickettii	Trypticase Soy Agar	+	-
Sporothrix	Trypticase Soy Agar	72 hr / room temp	-
Serratia liquefaciens	Trypticase Soy Agar	+	-
Escherichia coli	Trypticase Soy Agar	+	-
Aeromonas hydrophilia	Trypticase Soy Agar	+	-
Acinetobacter baumannii	Trypticase Soy Agar	+	
Corynebacterium ammoniagenes	Trypticase Soy Agar	+	-

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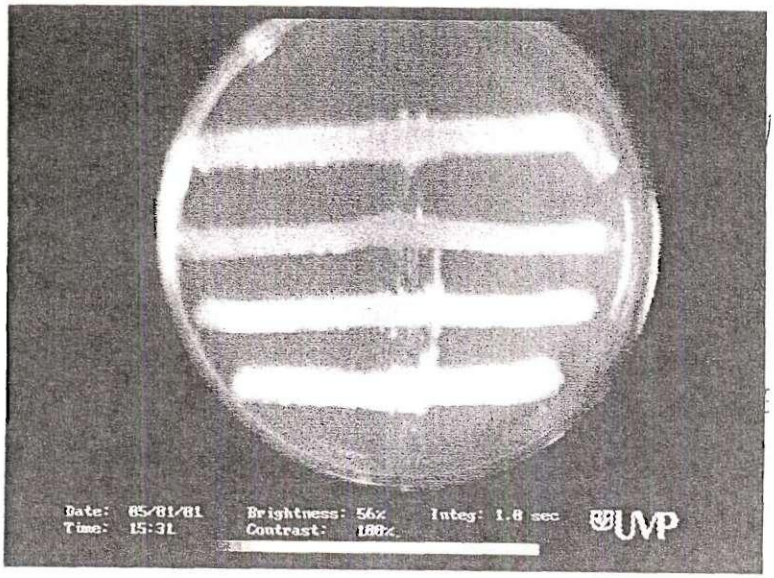

FIG. 14. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms: **1.)** *Alcaligenes faecalis* (-) **2.)** *Pseudomonas stutzer* (-) **3.)** *Providencia rettgeri* (-) **4.)** *Klebsiella pneumoniae* (-) Growth inhibition (+), No growth inhibition (-).

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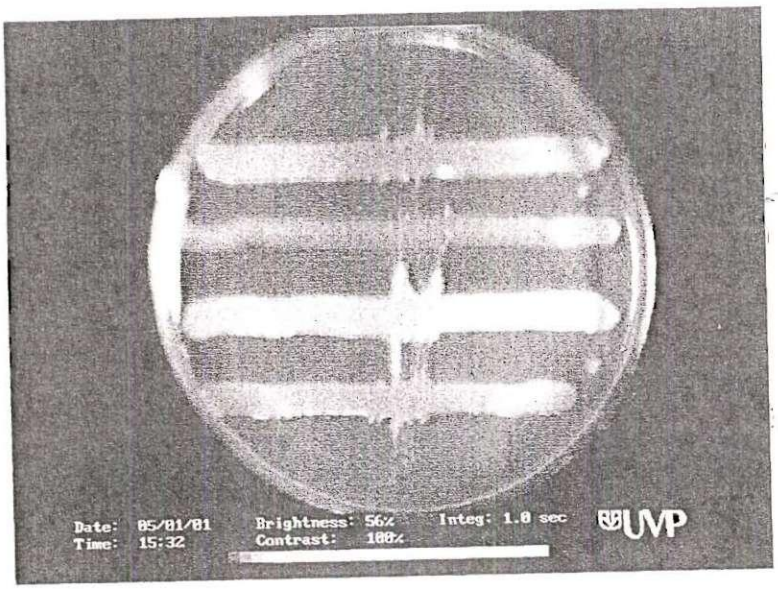

FIG. 15. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms: **1.)** *Pseudomonas aeruginosa* (-) **2.)** *Candida guillermondi* (-) **3.)** *Burkholderia gepacia* (-) **4.)** *Proteus vulgaris* (-) Growth inhibition (+), No growth inhibition (-).

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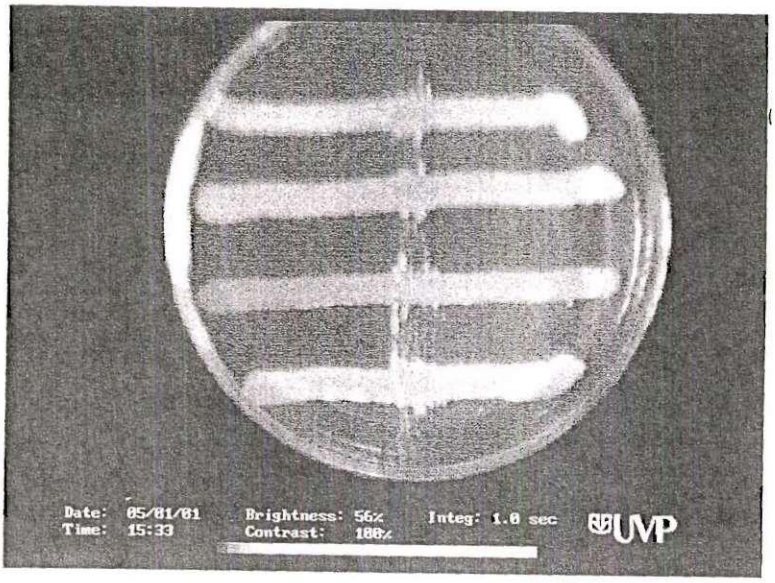

FIG. 16. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms:
1.) *Enterobacter aerogenes* (-) 2.) *Shewanella putrefaciens* (-) 3.) *Ralstonia pickettii* (-)
4.) *Serratia liquefaciens* (-) Growth inhibition (+), No growth inhibition (-).

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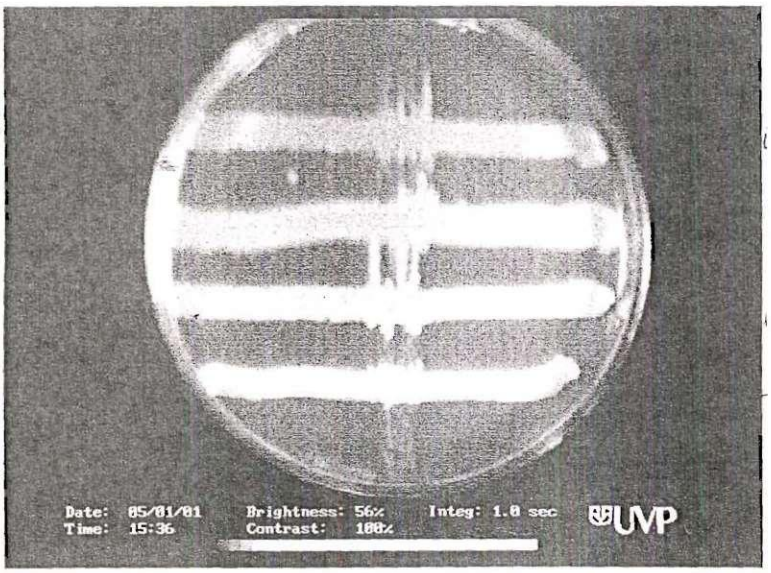

FIG. 17. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms:
1.) *Escherichia coli* (-) 2.) *Aeromonas hydrophilia* (-) 3.) *Acinetobacter baumannii* (-)
4.) *Corynebacterium ammoniagenes* (-) Growth inhibition (+), No growth inhibition (-).

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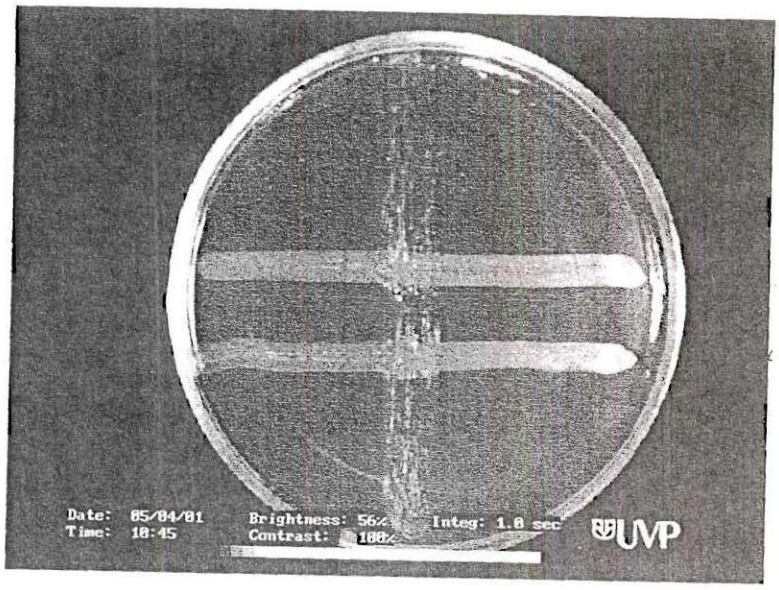

FIG. 18. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms: 1.) *Rhodoturula glutinis* (no growth) 2.) *Sphingobacterium spiritivorum* (-) 3.) *Chryzeobacterium meningosepticum* (-) Growth inhibition (+), No growth inhibition (-)

A

1

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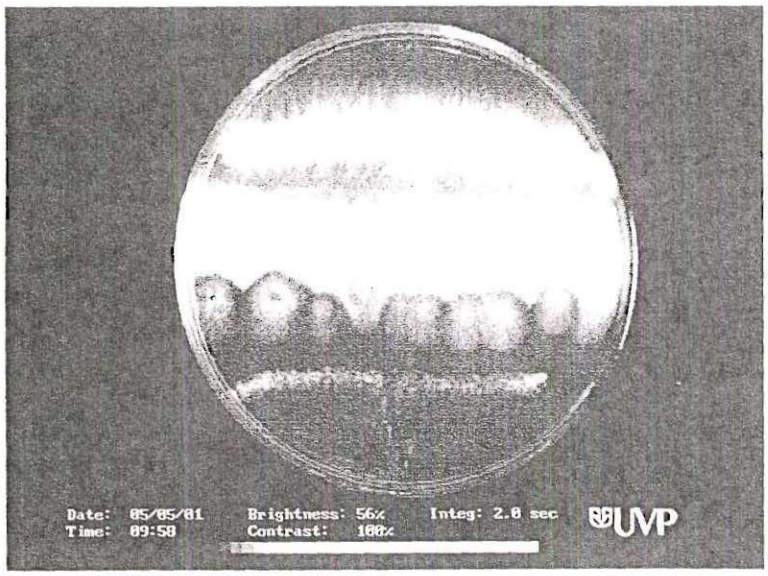

FIG. 19. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms: 1.) *Geotrichum candidum* (-) 2.) *Trichoderma viride* (-) 3.) *Aspergillus* (-) 4.) *Sporothrix* (-) Growth inhibition (+), No growth inhibition (-).

A

1

2

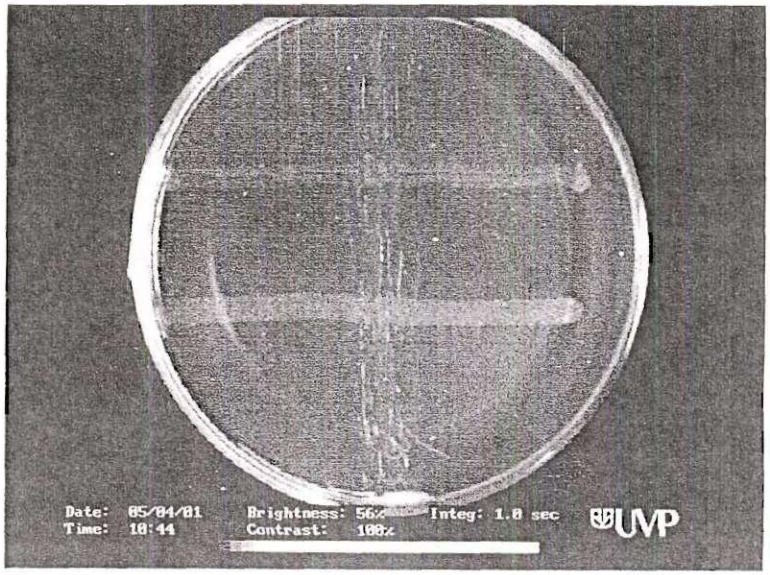

FIG. 20. **A.)** Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms: **1.)** *Gluconoacetobacter liquefaciens* (-) **2.)** *Gluconoacetobacter liquefaciens* (-) Growth inhibition (+), No growth inhibition (-).

A

1

2

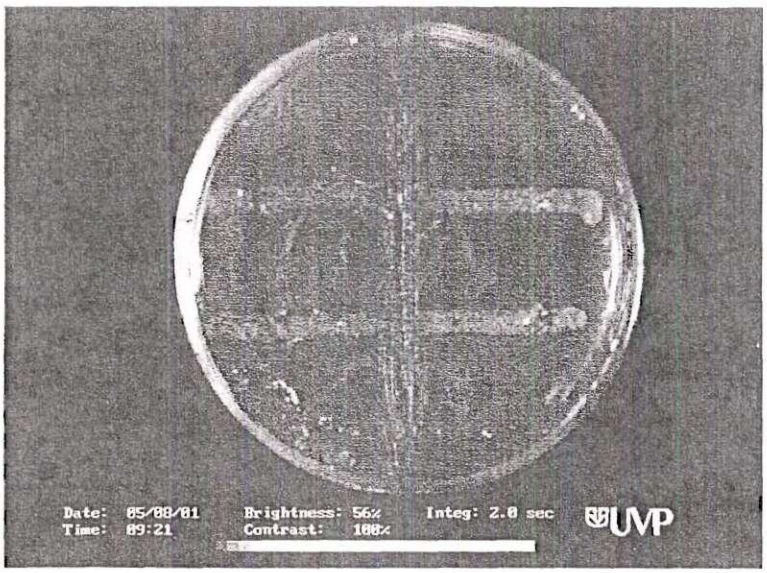

FIG. 21. A.) Center-Streak Organism: *Candida tropicalis*. Cross-Streak Organisms: **1.)** *Methylobacterium extorquens* (-) **2.)** *Methylobacterium extorquens* (-) Growth inhibition (+), No growth inhibition (-).

Table 8. **Results for *Pseudomonas aeruginosa* Cross-Streak Interactions**

Cross-Streak Organism	Media	Incubation for 24hr at 37°C or otherwise indicated	Inhibition (+) or No inhibition (-)
Alcaligenes faecalis	Trypticase Soy Agar	+	-
Pseudomonas stutzer	Trypticase Soy Agar	+	-
Providencia rettgeri	Trypticase Soy Agar	+	-
Gluconoacetobacter liquefaciens	Potato Dextrose Agar	72 hr / room temp	+
Klebsiella pneumoniae	Trypticase Soy Agar	+	+
Rhodoturula glutinis	Trypticase Soy Agar	No growth	
Geotrichum candidum	Trypticase Soy Agar	48hr / 37°C	-
Candida tropicalis	Trypticase Soy Agar	+	+
Candida guillermondi	Trypticase Soy Agar	+	-
Sphingobacterium spirivitivorum	Trypticase Soy Agar	+	-
Burkholderia gepacia	Trypticase Soy Agar	+	-
Proteus vulgaris	Trypticase Soy Agar	+	+
Enterobacter aerogenes	Trypticase Soy Agar	+	-
Methylobacterium exterquens	Trypticase Soy Agar	No growth	
Trichoderma viride	Trypticase Soy Agar	48 hr / 37°C	-
Shewanella putrefaciens	Trypticase Soy Agar	+	-
Chryzeobacterium meningosepticum	Trypticase Soy Agar	+	-
Aspergillus	Trypticase Soy Agar	48 hr / 37°C	-
Ralstonia picketti	Trypticase Soy Agar	+	+
Sporothrix	Trypticase Soy Agar	72 hr / room temp	-
Serratia liquefaciens	Trypticase Soy Agar	+	-
Escherichia coli	Trypticase Soy Agar	+	-
Aeromonas hydrophilia	Trypticase Soy Agar	+	+
Acinetobacter baumannii	Trypticase Soy Agar	No growth	
Corynebacterium ammoniagenes	Trypticase Soy Agar	+	+

A

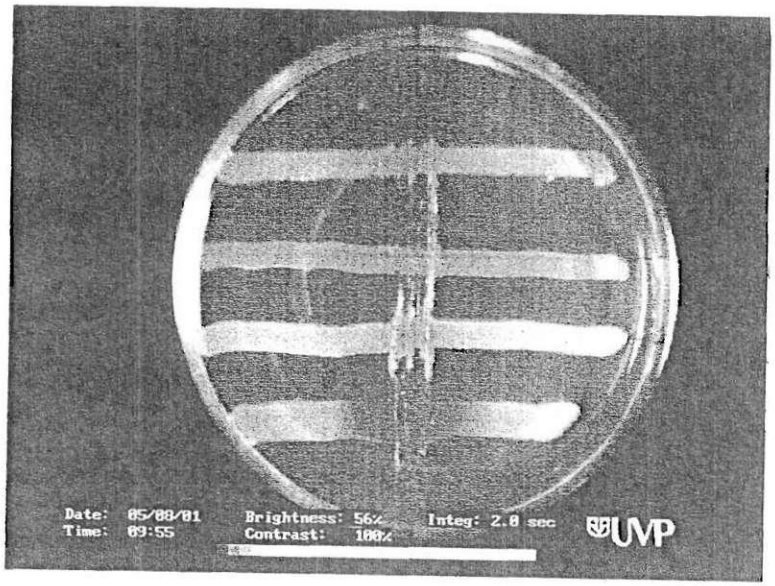

FIG. 22. **A.)** Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: **1.)** *Alcaligenes faecalis* (-) **2.)** *Pseudomonas stutzer* (-) **3.)** *Providencia rettgeri* (-) **4.)** *Klebsiella pneumoniae* (+) Growth inhibition (+), No growth inhibition (-).

A

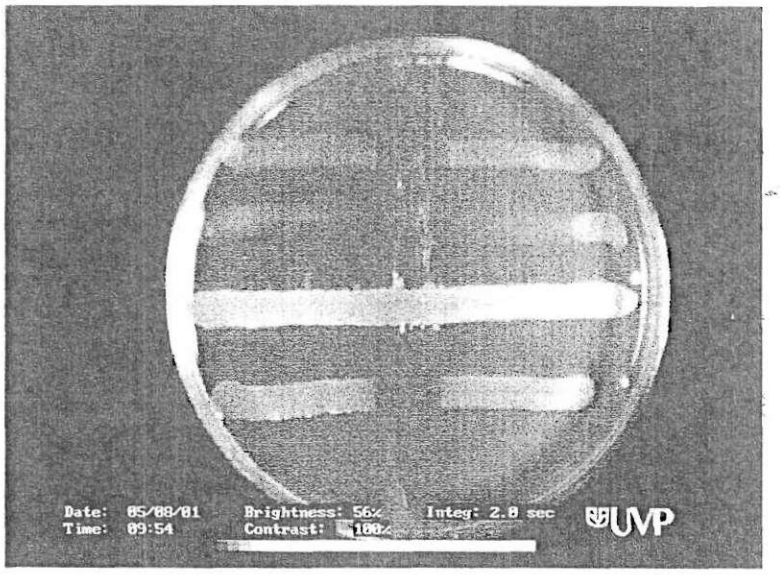

FIG. 23. **A.)** Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: **1.)** *Candida tropicalis* (+) **2.)** *Candida guillermondi* (-) **3.)** *Burkholderia gepacia* (-) **4.)** *Proteus vulgaris* (+) Growth inhibition (+), No growth inhibition (-).

A

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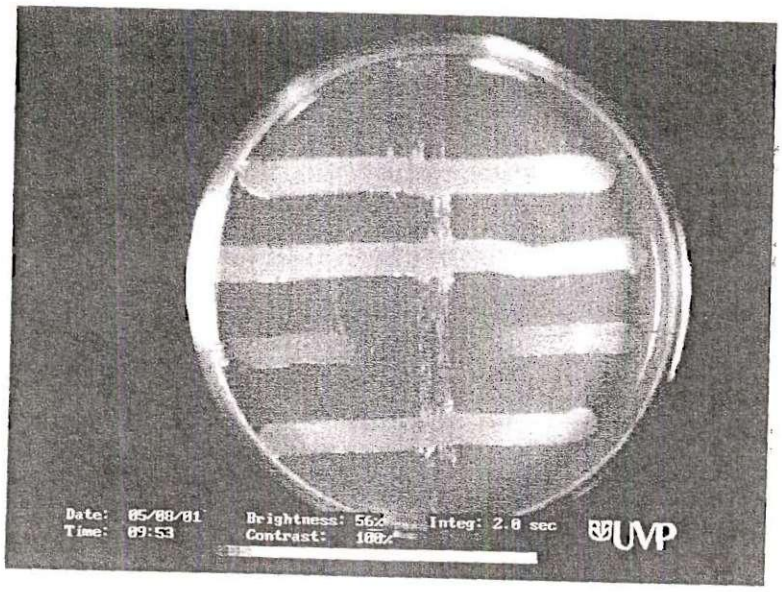

FIG. 24. A.) Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: **1.)** *Enterobacter aerogenes* (-) **2.)** *Shewanella putrefaciens* (-) **3.)** *Ralstonia pickettii* (+) **4.)** *Serratia liquefaciens* (-) Growth inhibition (+), No growth inhibition (-).

A

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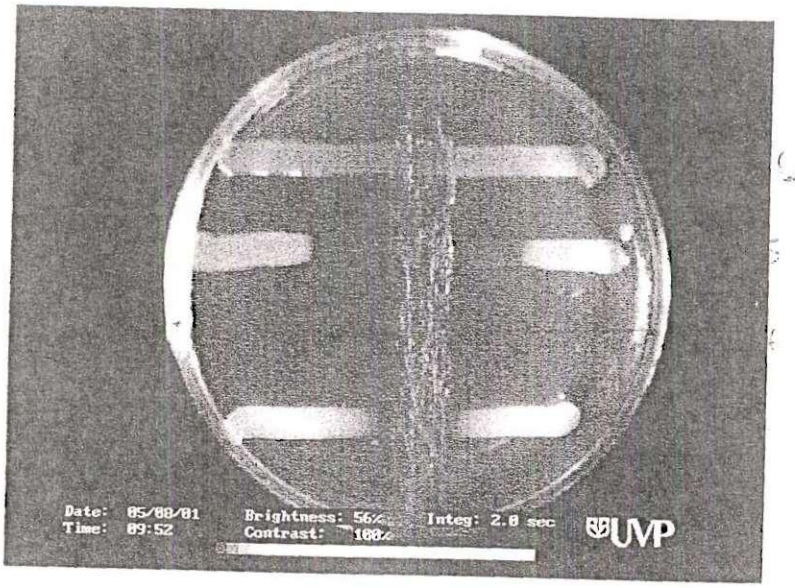

FIG. 25. A.) Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: **1.)** *Escherichia coli* (-) **2.)** *Aeromonas hydrophilia* (+) **3.)** *Acinetobacter baumannii* (No growth) **4.)** *Corynebacterium ammoniagenes* (+) Growth inhibition (+), No growth inhibition (-).

A

1

2

3

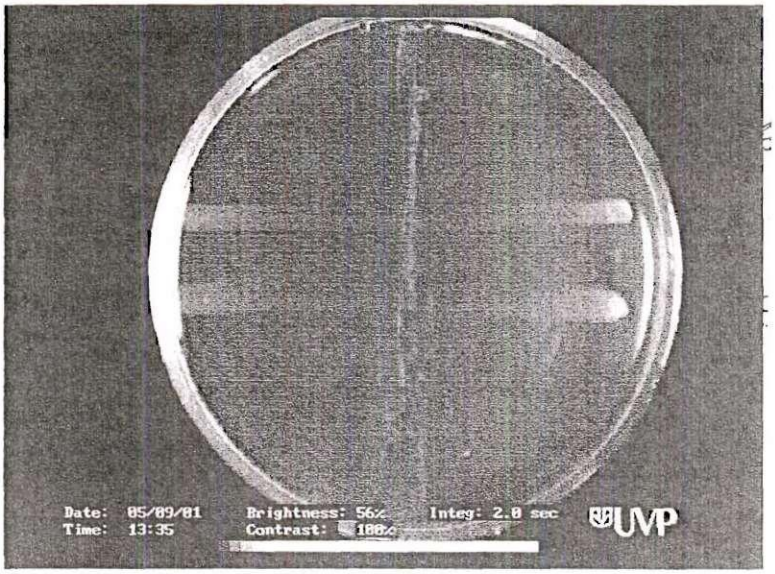

FIG. 26. **A.)** Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: **1.)** *Rhodoturula glutinis* (no growth) **2.)** *Sphingobacterium spiritivorum* (-) **3.)** *Chryzeobacterium meningosepticum* (-) Growth inhibition (+), No growth inhibition (-)

A

1

2

3

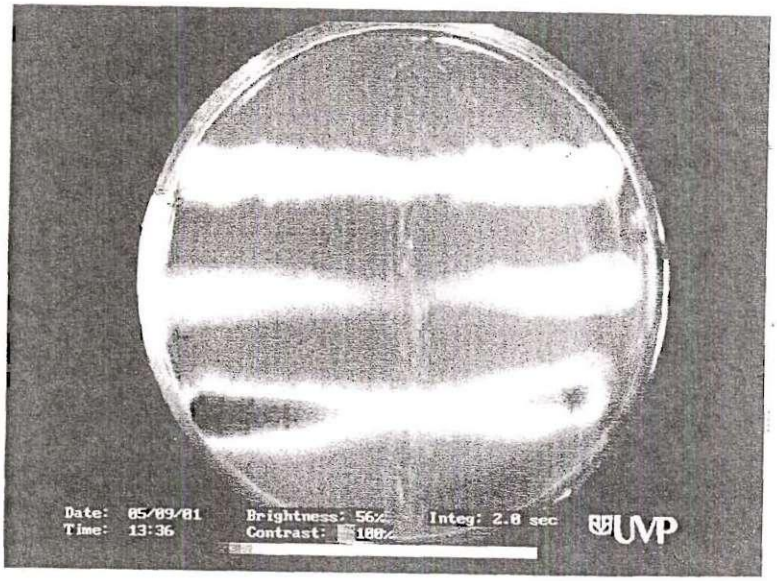

FIG. 27. **A.)** Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: **1.)** *Geotrichum candidum* (-) **2.)** *Trichoderma viride* (-) **3.)** *Aspergillus* (-) Growth inhibition (+), No growth inhibition (-).

A

1

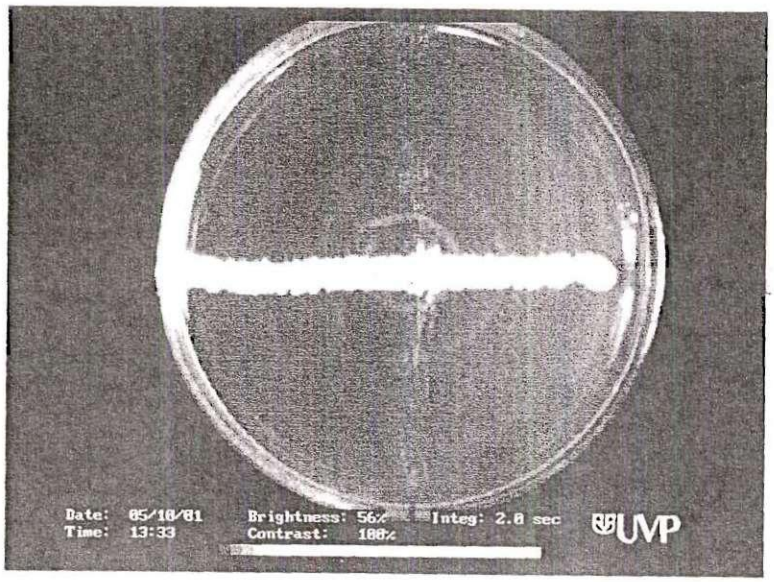

FIG. 28. A.) Center-Streak Organism: *Pseudomonas aeruginosa*. Cross-Streak Organisms: 1.) *Sporothrix* (-) Growth inhibition (+), No growth inhibition (-).

A

1

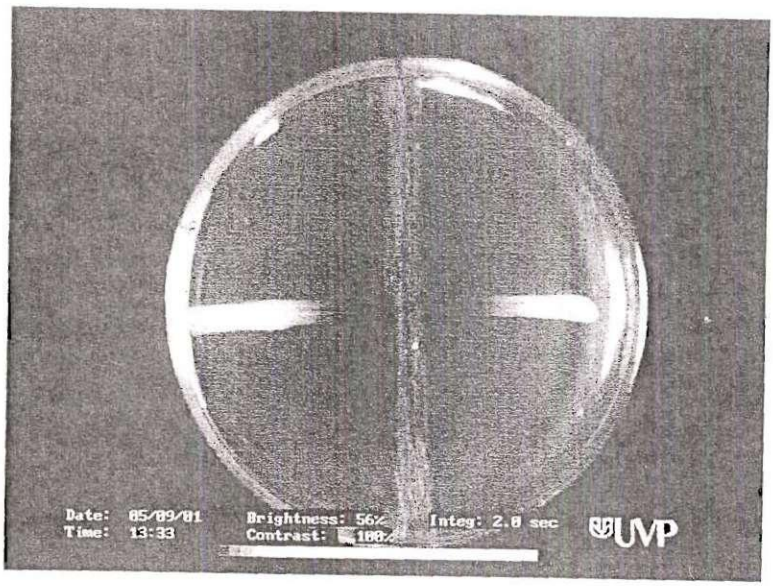

FIG. 29. A.) Center-Streak Organism: *Pseudomonas aeruginosa* Cross-Streak Organisms: 1.) *Gluconoacetobacter liquefaciens* (-). Growth inhibition (+), No growth inhibition (-).

Table 9.**Results for *Corynebacterium ammoniagenes* Cross-Streak Interactions**

Cross-Streak Organism	Media	Incubation for 24hr at 37°C or otherwise indicated	Inhibition (+) or No inhibition (-)
Alcaligenes faecalis	Trypticase Soy Agar	+	-
Pseudomonas stutzer	Trypticase Soy Agar	+	-
Providencia rettgeri	Trypticase Soy Agar	+	-
Gluconoacetobacter liquefaciens	*C. ammoniagenes* does not grow on potato dextrose		
Klebsiella pneumoniae	Trypticase Soy Agar	+	-
Rhodoturula glutinis	Trypticase Soy Agar	No growth	
Geotrichum candidum	Trypticase Soy Agar	48hr / 37°C	-
Candida tropicalis	Trypticase Soy Agar	+	-
Pseudomonas aeruginosa	Trypticase Soy Agar	+	-
Candida guillermondi	Trypticase Soy Agar	+	-
Sphingobacterium spirivitivorum	Trypticase Soy Agar	+	-
Burkholderia gepacia	Trypticase Soy Agar	+	-
Proteus vulgaris	Trypticase Soy Agar	+	-
Enterobacter aerogenes	Trypticase Soy Agar	+	-
Methylobacterium exterquens	Trypticase Soy Agar	No growth	
Trichoderma viride	Trypticase Soy Agar	48 hr / 37°C	-
Shewanella putrefaciens	Trypticase Soy Agar	+	-
Chryzeobacterium meningosepticum	Trypticase Soy Agar	+	-
Aspergillus	Trypticase Soy Agar	48 hr / 37°C	-
Ralstonia pickettii	Trypticase Soy Agar	+	-
Sporothrix	Trypticase Soy Agar	72 hr / room temp	-
Serratia liquefaciens	Trypticase Soy Agar	+	-
Escherichia coli	Trypticase Soy Agar	+	-
Aeromonas hydrophilia	Trypticase Soy Agar	+	-
Acinetobacter baumannii	Trypticase Soy Agar	No growth	

A

1

2

3

4

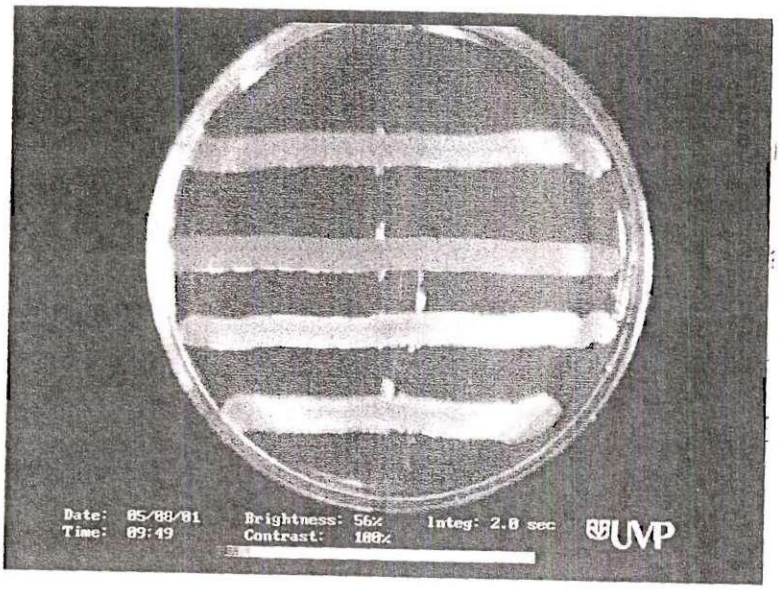

FIG. 30. **A.)** Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: **1.)** *Alcaligenes faecalis* (-) **2.)** *Pseudomonas stutzer* (-) **3.)** *Providencia rettgeri* (-) **4.)** *Klebsiella pneumoniae* (-) Growth inhibition (+), No growth inhibition (-).

A

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2

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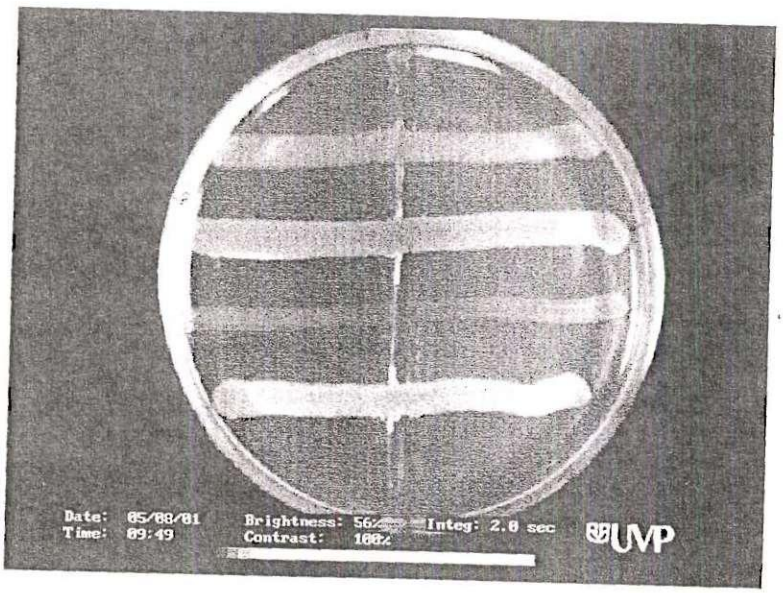

FIG. 31. **A.)** Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: **1.)** *Candida tropicalis* (-) **2.)** *Pseudomonas aeruginosa* (-) **3.)** *Candida guillermondi* (-) **4.)** *Burkholderia gepacia* (-) Growth inhibition (+), No growth inhibition (-).

A

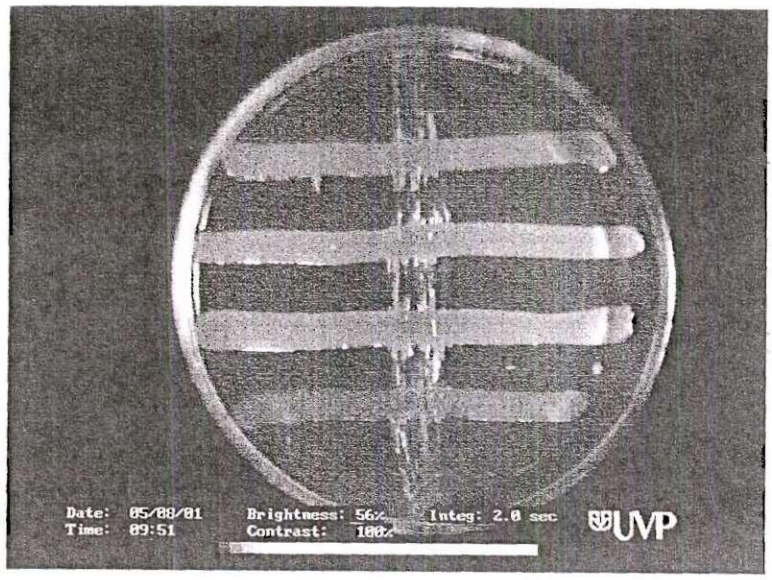

FIG. 32. **A.)** Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: **1.)** *Proteus vulgaris* (-) **2.)** *Enterobacter aerogenes* (-) **3.)** *Shewanella putrefaciens* (-) **4.)** *Ralstonia pickettii* (-) Growth inhibition (+), No growth inhibition (-).

A

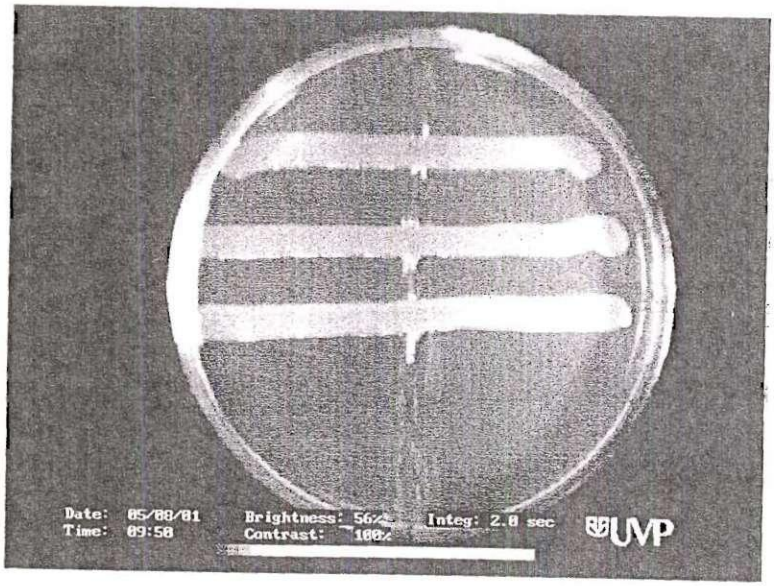

FIG. 33. **A.)** Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: **1.)** *Serratia liquefaciens* (-) **2.)** *Escherichia coli* (-) **3.)** *Aeromonas hydrophilia* (-) **4.)** *Acinetobacter baumannii* (No growth). Growth inhibition (+), No growth inhibition (-).

1

2

3

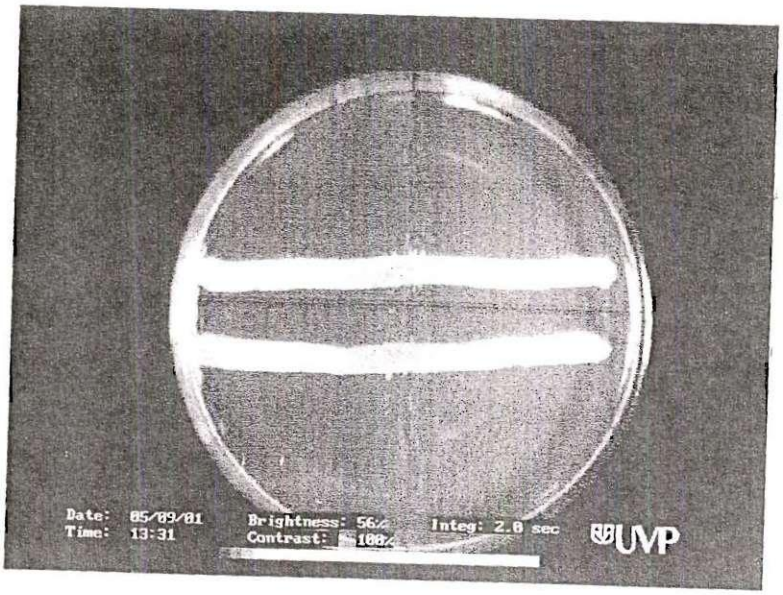

FIG. 34. A.) Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: 1.) *Rhodoturula glutinis* (no growth) 2.) *Sphingobacterium spiritivorum* (-) 3.) *Chryzeobacterium meningosepticum* (-) Growth inhibition (+), No growth inhibition (-)

A

1

2

3

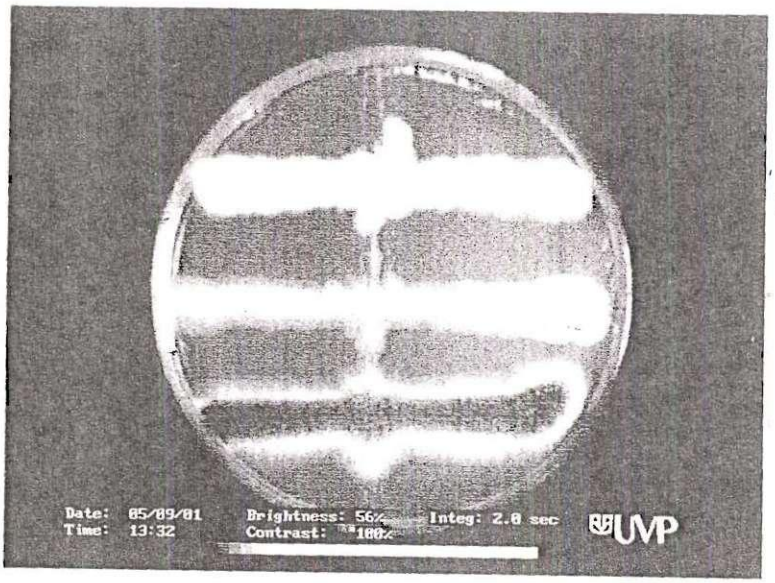

FIG. 35. A.) Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: 1.) *Geotrichum candidum* (-) 2.) *Trichoderma viride* (-) 3.) *Aspergillus* (-) Growth inhibition (+), No growth inhibition (-).

A

1

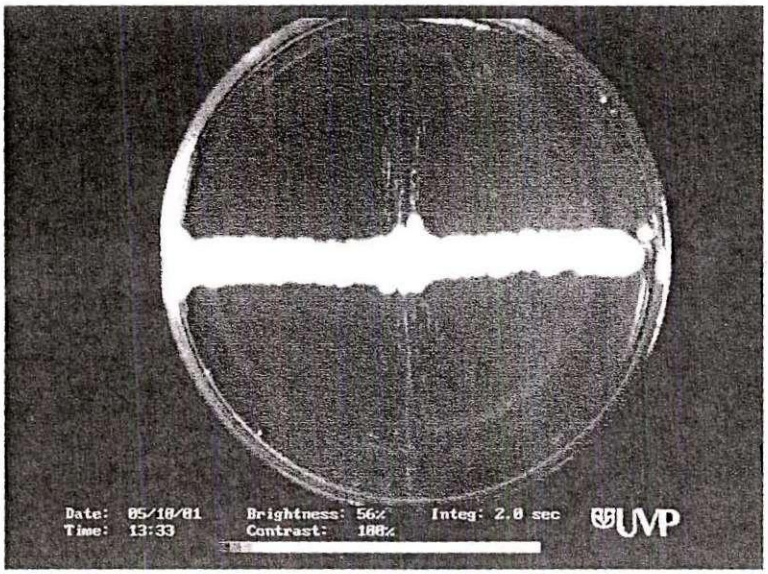

FIG. 36. **A.)** Center-Streak Organism: *Corynebacterium ammoniagenes*. Cross-Streak Organisms: **1.)** *Sporothrix* (-) Growth inhibition (+), No growth inhibition (-).

Table 10 **MICROBIAL INTERACTION WITH EMULSION**

Emulsion: AIRFLEX 400 (Before Biocide), Lot # C – 145

Test Organism: *Gluconoacetobacter liquefaciens*

Date	Week #	pH	Appearance	Odor	Physical Separation	CFU/ml
5/08/01	0	4.6	0 = No change	0 = None	0 = None	$2.8 * 10^6$
5/15/01	1	5.0	0 = No change	0 = None	+2 = Creamy	$3.7 * 10^7$
5/25/01	2	6.0	0 = No change	+4 = Atypical	+2 = Creamy	$1.3 * 10^8$
5/31/01	3	6.3	0 = No change	+4 = Atypical	+2 = Creamy	In progress
	4					
	5					
	6					
	7					
	8					

Table11 MICROBIAL INTERACTION WITH EMULSION

Emulsion: AIRFLEX 400 (Before Biocide), Lot # C – 145

Test Organism: *Candida tropicalis*

Date	Week #	pH	Appearance	Odor	Physical Separation	CFU/ml
5/08/01	0	4.6	0 = No change	0 = None	0 = None	$7.1 * 10^5$
5/15/01	1	4.5	0 = No change	0 = None	0 = None	0
5/25/01	2	4.5	0 = No change	0 = None	0 = None	0
5/31/01	3	4.45	0 = No change	0 = None	0 = None	0
	4					
	5					
	6					
	7					
	8					

Table12 **MICROBIAL INTERACTION WITH EMULSION**

Emulsion: AIRFLEX 400 (Before Biocide), Lot # C – 145

Test Organism: *Alcaligenes faecalis*

Date	Week #	pH	Appearance	Odor	Physical Separation	CFU/ml
5/16/01	0	4.51	0 = No change	0 = None	0 = None	$1.3 * 10^7$
5/24/01	1	4.5	0 = No change	0 = None	0 = None	0
5/31/01	2	4.48	0 = No change	0 = None	0 = None	0
	3					
	4					
	5					
	6					
	7					
	8					

Table 13 **MICROBIAL INTERACTION WITH EMULSION**

Emulsion: AIRFLEX 400 (Before Biocide), Lot # C – 145

Test Organism: *Acinetobacter baumannii*

Date	Week #	pH	Appearance	Odor	Physical Separation	CFU/ml
5/16/01	0	4.51	0 = No change	0 = None	0 = None	$2.1 * 10^7$
5/24/01	1	4.55	0 = No change	0 = None	0 = None	0
5/31/01	2	4.48	0 = No change	0 = None	0 = None	0
	3					
	4					
	5					
	6					
	7					
	8					

Table 14 **MICROBIAL INTERACTION WITH EMULSION**

Emulsion: AIRFLEX 400 (Before Biocide), Lot # C – 145

Test Organism: *Aspergillus*

Date	Week #	pH	Appearance	Odor	Physical Separation	CFU/ml
5/16/01	0	4.51	0 = No change	0 = None	0 = None	N/A
5/25/01	1	4.5	0 = No change	0 = None	0 = None	N/A
5/31/01	2	4.5	0 = No change	0 = None	0 = None	N/A
	3					
	4					
	5					
	6					
	7					
	8					

Table 15 **MICROBIAL INTERACTION WITH EMULSION**

Emulsion: AIRFLEX 400 (Before Biocide), Lot # C – 145

Test Organism: *Geotrichum candidum*

Date	Week #	pH	Appearance	Odor	Physical Separation	CFU/ml
5/16/01	0	4.51	0 = No change	0 = None	0 = None	N/A
5/25/01	1	4.5	0 = No change	0 = None	0 = None	N/A
5/31/01	2.	4.48	0 = No change	0 = None	0 = None	N/A
	3					
	4					
	5					
	6					
	7					
	8					

"Statistical Analysis of Bacterial Counts"

Calculation of Mean (Average)

In order to calculate the average number of colonies per plate, χ, the following method is used:

$$\chi = [\Sigma(x_i)] / n$$

Calculation of the Standard Deviation

This value is the number that can give an idea of the average range of values that stray from the mean. In other words, if a calculation of the mean is found to be 10 but it has a standard deviation of 2 then one can say that for the most the part values found range between 8-12. In totality, the mean and the standard deviation are reported together as 10 ± 2.

$$SD = \sigma = \sqrt{[(\Sigma (x_i - \chi)^2)/(n-1)]}$$

x_i = number of individual colonies found on one plate

n = number of plates (*note this will be the number of plates for ONE series. In other words, you would count the number of plates for that one dilution factor. In our case there will be n=3 plates for the 10^{-3} dilution.

Calculation of the Confidence Interval of 95%

In this situation, we will never truly know the exact number of colonies in our sample. Thus, by taking counts of dilutions spread out on plates, we are merely taking an *estimate* of the true mean. In such a situation, we must use the following procedure to calculate a 95% Confidence Interval. It should be noted that a 95% Confidence Interval would be defined as:

> *"95% of the confidence interval constructed in this manner from repeated samples of size n from this reference population will contain the true mean, μ."*

To construct a 95% Confidence Interval, the following formula is used:

$$\chi \pm [\ (t_{(n-1),\ \%CI} \cdot\ SE\ ]$$

For a 95% confidence interval with a value of n = 3, and a Degree of Freedom, (N-1), is = 2

$$\chi \pm [\ (4.303 \cdot SE\ ]$$

SE = standard error = the standard deviation of our estimate = [б / √ n]

Note that the value 4.303 was found using TABLE A.

TABLE A: T-Distribution Table of Values

Degrees if Freedom, (N-1)	0.90	0.95	0.975	0.99
	90% CI	95% CI	95% CI	99% CI
1	3.078	6.314	12.706	31.821
2	1.886	2.920	4.303	6.965
3	1.638	2.353	3.182	4.541
4	1.533	2.132	2.776	3.747

CI = Confidence Interval

Appendix II

MICROBIAL INTERACTIONS WITH EMULSIONS (INTERPRETATION OF QUALITATIVE CHANGES)

I. Odor:

None	----> 0
Sweat Sock	---> +
Putrid	---> ++
Acetone	----> +++
Atypical	----> ++++

II. Appearance:

No Change	----> 0
Gray	----> +
Pink	----> ++
Light Brown (Creamy)	----> +++
Atypical	----> ++++

III. Phase Separation (depth of 1 to 2 mm)

None	----> 0
Brown	-----> +
Creamy	-----> ++
Atypical	-----> +++

BRIEF DESCRIPTIONS OF ORGANISMS USED IN EMULSION STUDIES

Alcaligenes faecalis

Gram -ve, catalse +ve, and oxidase +ve rods. Metabolism is respiratory. All strains can grow chemoorganotrophically on e.g acetate, lactate, malate or succinate. Most strains do not use carbohydrates but some can form acid from glucose and/or xylose. Optimum growth media is peptone containing media and blood agar.

Pseudomonas stutzeri

Gram -ve, aerobic, chemoorganotrophic or facultatively chemolithotropic bacteria. Nutritionally and metabolically highly versatile. Hydrocarbons can be metabolized by some strains. Some species produce bacteriocins. Optimum growth temperature is 28 - 30°C, and are generally inhibited at or below pH 4.5.

Providencia rettgeri

A genus of gram -ve rods. They can metabolize mannose and a few sugar alcohols. Nicotinic and pantothenic acids are not required for growth.

Gluconoacetobacter liquefaciens

A genus of rod shaped gram -ve bacteria of *Acetobacteriaceae*. The organisms occur e.g in fruits and flowers and are used for production of monobactams and in sorbose fermentation. Ketogenic growth occurs on polyalcohol substrates. All strains require growth factors which may include pantothenate, niacin, thiamine, PABA. GYC media. Optimum growth temperature 25 - 30°C. pH 4-6. Slow growing 3-10 days.

Klebsiella pneumoniae

Gram -ve rods of genus *Enterobacteriaceae*. The cells are capsulated and form mucoid colonies in carbohydrate -rich media. Growth in KCN media.

Rhodoturula glutinis

Class *Hyphomycetes*. A genus of "imperfect" yeast. Metabolism is strictly respiratory.

Geotrichum candidum

Genus of fungi. Class *Hyphomycetes.*

Candida tropicalis

A large heterogeneous genus of yeast-like or dimorphic imperfect fungi class *Hyphomycetes.*

Pseudomonas aeruginosa

Gram -ve, aerobic, chemoorganotrophic or facultatively chemolithotropic bacteria. Nutritionally and metabolically highly versatile. . Hydrocarbons can be metabolized by some strains. Some species produce bacteriocins. Optimum growth temperature is 28 - 30°C, and are generally inhibited at or below pH 4.5

Candida guillermondi.

A large heterogeneous genus of yeast-like or dimorphic imperfect fungi class *Hyphomycetes.*

Sphingobacterium spiritivorum.

A genus proposed for sphingolipid-containing strains of *Flavobacterium*. Gram -ve, aerobic, chemoorganotrphic bacteria. Metabolism is respiratory (oxidative). Most grow on nutrient agar, but may require enrichment such as casein, glucose or yeast extract.

Burkholderia gepacia

Also known as *Pseudomonas cepacia.* Gram -ve, aerobic, chemoorganotrophic or facultatively chemolithotropic bacteria. Nutritionally and metabolically highly versatile. Non-fluorescent pigments may form. Optimum growth temp. 30-35°C.

Proteus vulgaris

Gram -ve bacteria. Most strains swarm at 37°C. Some strains may form a uniform film of growth.TSI +ve, Indole +ve, maltose +ve, mannose and sugar alcohol are not attacked.

Enterobacter aerogenes

Gram -ve rods of *Enterobacteriaceae*. Facultatively anaerobic. Optimum growth temp is 30^0C. Metabolism is chemoorganotrophic and may be respiratory or fermentative depending on the conditions.

Cladosporium

A genus of fungi , class *Hyphomycetes*. Causes paint spoilage.

Methylobacterium extorquens

A genus of facultatively methylotrophic and methanotrophic, rod-shaped bacteria which can alternatively use glucose and other complex substrates as sole source of carbon and energy. Pigments are formed by some strains.

Trichoderma viride

A genus of fungi, class *Hyphomycetes*. Grow rapidly and can use a wide range of substrates. Many are antagonistic to other fungi e.g by forming antibiotics and/or by competing for nutrients.

Shewanella putrefaciens

Include strains of barophilic bacteria. Capable of anaerobic respiration by ferric iron reduction.

Chryzeobacterium meningosepticum

Chryseobacterium meningosepticum, formerly known as Flavobacterium meningosepticum and CDC II-a, is a gram-negative rod widely distributed in nature

Aspergillus

Fungi of class *Hyphomycetes*. They can use a wide range of substrates as nutrients. Some species may cause deterioration in various types of materials including paint spoilage.

Ralstonia pickettii

Gram -ve rod. Facultative chemolithoautotropic.

Sporothrix

A genus of dimorphic fungi of *Hyphomycetes*. The yeast form occurs in culture at 37°C.

Serratia liquefaciens

Formerly *Enterobacter liquefaciens*. Gram -ve bacteria of *Enterobacteriaceae*. Optimum growth temp 30°C.

Escherichia coli

Gram -ve bacteria of *Enterobacteriaceae*.

Aeromonas hydrophilia

A genus of gram -ve bacteria of *Vibronaceae*. Rods or coccobacilli. A range of sugars and organic acids can be used as carbon source.

Acinetobacter baumannii.

A genus of oxidase-negative, strictly aerobic, gram -ve bacteria. Family *Neisseriaceae*. Metabolism is respiratory. Most strains can grow on mineral salts medium containing e.g. acetate, ethanol or lactate. Some strains can degrade benzoate and alicyclic compounds.

Corynebacterium ammoniagenes

A genus of gram +ve, aerobic, facultatively anaerobic, chemoorganotrophic, bacteria. Metabolism can be oxidative or fermentative.

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#'s 4 + 5

FINAL REPORT

FOURTH QUARTER PROGRESS REPORT

(SECOND-YEAR GRANT PERIOD)

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIONS: BEHAVIOR OF MICROORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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Edmonds, P., Second-Year Grant Period (Fourth Quarter Report, 11/21/02)

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I. EXECUTIVE SUMMARY

Our studies over the past two years have generated a large quantity of data on interactions among several microorganisms found to contaminate polymer emulsions. During the second year, we focused our studies on dominant organisms that exhibited inhibitory actions against other emulsion isolates in cross-streak experiments, and in liquid culture. First, we performed many experiments to determine which organisms would grow in polymer emulsions, and which organisms would grow in a Mineral Salts Medium, supplemented with an emulsion component.

Experiments performed to establish "growth curves" for GABL in Airflex 400 (using several different variables) were described in the **Third Quarter Report (2nd-Year Grant Period).** Data from those experiments, enabled us to identify the **"lag phase of growth"** and the **"exponential phase of growth"** (i.e., specific time intervals within the grow cycle) for GABL. Those data provided a foundation for experiments that were conducted during this fourth quarter reporting period. Those findings demonstrated by photographs, and by quantitative cell counts provide evidence that dominant species (GABL and *Pseudomonas aeruginosa*) have the potential to regulate population density in laboratory experiments.

Experiments performed during this **Final Report (Fourth Quarter , 2nd -Year Grant Period)** are described (summarized) in several broad categories.

A) We measured growth of *Gluconoacetobacter liquefaciens* (GABL), *Pseudomonas aeruginosa,* and *Candida tropicalis* in a Mineral Salts Medium, supplemented with an emulsion component: Polystep B27, Airvol 523, or Colloid 675.

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Results show GABL and *P. aeruginosa* did not grow in the presence of Polystep B27 (See Table 1 and Figure 1). No viable cells were detected for either of these organisms after 72 hrs. These results are consistent with data previously reported for these organisms (See Table 9) in our **Second Quarter Report, 2nd-Year Grant Period (5/8/02).**

In another experiment, GABL was evaluated for growth in the Mineral Salts Medium, supplemented with Airvol 523. Our results show that GABL grew well, with cell numbers increasing approximately two logs in three days (Table 5 and Figure 3).

In contrast to growth responses for GABL and *P. aeruginosa* shown in Table 1 and Figure 1, *C. tropicalis* grew well in the Mineral Salts Medium, supplemented with Polystep B27 (Table 3, Figure 2).

B) In our **Third Quarter Report, 2nd-Year Grant Period (7/24/02)**, we evaluated GABL for growth in Airflex 400 using several variables, but we did not measure the growth of *C. tropicalis.* During this Fourth Quarter reporting period, we measured the growth of *C. tropicalis*, using those same variables, and the results are shown in Table 2. No viable cells were detected after 24 hrs. We measured growth at 24-hr intervals for four days, and terminated that experiment.

C) We performed several "new" experiments aimed at generating some definitive information that would provide some insights on the nature of the secreted inhibitory substance from GABL and *P. aeruginosa*. Presumptive information generated from cross-streak experiments, and from cell-free extracts indicated that such inhibitory Activities could result from **"lytic phages", "prophages, "bacteriocins" or "killer**

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proteins". Our results were not definitive. We used classical "rapid methods" to demonstrate activities for such substances. Among factors that may account for our lack of getting positive results are: (i) various kinds of inhibitory substances synthesized in cells are encoded by genes that are regulated in response to density-dependent cell numbers, called **"quorum sensing molecules"**, and (ii) antibiotics and some bacteriocins are excreted as **secondary metabolites** (during the stationary phase of growth), and (iii) some inhibitory polypepides are excreted from cells as **inactive proteins,** that are activated by extracellular proteases, and (iv) our cell-free extracts may be inhibitory, but are present in concentrations too low for detection by assays used in our studies

Physical data from experiments conducted by Dr. Schork are shown in a Supplement, following reference.

II. OBJECTIVES OF EXPERIMENTS DESCRIBED IN THIS REPORT

Experiments described in this report are divided into two broad categories: (i) Growth-response experiments of organisms in emulsions and/or emulsion components, performed by Dr. Edmonds' group, and (ii) Physical properties of performed in the School of Chemical Engineering by Dr. Schork's group.

I. Growth Measurements of Microorganisms and Studies on Inhibitory Substances.

A. To determine the growth response of *Gluconoacetobacter liquefaciens* (GABL), *Pseudomonas aeruginosa*, and *Candida tropicalis* in a Mineral Salt Medium supplemented with one of the following emulsion components in separate culture vessels: Polystep B27; Airvol 523; and Colloid 675.

B. To determine the growth response of *Candida tropicalis* in Airflex 400.

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C. To continue on-going experiments designed to evaluate the nature of excreted inhibitory substances produced by GABL and *Pseudomonas aeruginosa.*

II. To Measure Physical Parameters of Emulsions (Dr. Schork's Laboratory).

To measure physical characteristics of two emulsions (Airflex 400 and Airflex 192) to determine changes in some parameters (pH, viscosity, conductivity, insoluble fraction, mesh grit, particle radius, gel content) following inoculation with GABL and *Candida tropicalis* (separately). Nine samples were analyzed.

- Latex 1: Airflex 400 with GABL (8/22/01-10/10/01)
- Latex 2: Airflex 192 with GABL (11/01/01-1/23/01)
- Latex 3: Airflex 400 with GABL (11/01/01-1/23/01)
- Latex 4: Airflex 400 with GABL (2/04/02-2/25/02)
- Latex 5: Airflex 192 with GABL (2/04/02-2/25/02)
- Latex 6: Airflex 400 with *Canidida* (3/12/02-3/25/02)
- Latex 7: Airflex 192 with *Canidida* (3/12/02-4/23/02)
- Latex 8: Airflex 400 with GABL (5/28/02-6/17/02)
- Latex 9: Airflex 192 with GABL (5/28/02-6/17/02)
- Final and Control Analysis: Overview of all the latex samples

III. METHODS/PROCEDURES/PROTOCOLS

I. Growth Measurements of Microorganisms and Studies of Inhibitory Substances.

A. Protocols for "growth studies" were described in the **Third Quarter Report (First-Year Grant Period, 2001).**

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B. Protocols for "challenge experiments" to determine if organisms will grow (increase in cell numbers) in a Mineral Salt Medium (Bushnell-Hass Broth) supplemented with one of the following emulsion components were described in the **Second Quarter Report, First-Year Grant period (2001).** Emulsions components used in these experiments were: Polystep B27, Airvol 523, and Colloid 675.The Mineral Salts Medium was autoclaved separately, and allowed to cool. Then, the designated emulsion component was added aseptically. The final medium composition in each culture vessel was Bushnell-Hass Broth (90%) + a specified Emulsion component (10%). Then, a separate culture vessel was inoculated with a standardized suspension of "single species" of a organism: *Pseudomonas aeruginosa, Candida tropicalis* or GABL.

C. Protocols for evaluating the activity of cell-free excreted inhibitory substances produced by GABL and *Pseudomonas aeuginosa* were described in **Second Quarter Report (Second-Year Grant Period)**, briefly described with modifications.
We used data from growth-curves reported in our **Third-Quarter Report** that differentiated the exponential growth phase from the stationary growth phase of **GABL** cultivated in Airflex 400, and in Potato Dextrose Broth. Then for each culture, two samples were examined from different phases of growth: one was removed from **mid-exponential phase of growth,** and the other was removed from the **late stationary phase** of growth cycles. Samples were processed as follows: (i) cell suspensions (each sample size was 50 ml) from organisms were centrifuged to separate cells from supernatants: (ii) supernatants were centrifuged to obtain cell-free extracts were filtered using a 0.22-um pore size filter, and (iii) cell-free extracts diluted serially using two-fold

dilutions, and tested for inhibitory activity characteristic of bacteriophages (i.e., the "plaque assay") or characteristic of bacteriocins (i.e., polypeptide lysis).

Bacteriophages (phages) are viruses that infect only susceptible strains of bacteria, and may multiply within host bacterial cells through either of two alternative mechanisms: (i) the **lytic cycle** which ends with the lysis and death of the host bacterial cells, which can be detected by the formation of characteristics plaques on host cells, and (ii) the **lysogenic cycle**, during which the phage nucleic acid becomes integrated into the host bacterial chromosome as a **"prophage"**, and does not kill the host cell. Most of the prophage genes are suppressed. However, prophage genes can spontaneously be released from the host chromosome, form phage particles, and multiply through the lytic cycle.

IV. RESULTS

I. Growth Measurements of Microorganisms and Studies on inhibitory Substances.

Three organisms (GABL, *P. aeruginosa*, and *Candida tropicalis*) which have been isolated from contaminated emulsions were evaluated for their ability to grow (increase in numbers) in a mineral salts medium (Bushnell-Haas Broth) supplemented with each of the following emulsion components (separately). The growth response of GABL and *P.aeruginosa* in the mineral salts solution supplemented with Polystep B-27 are shown in Table 1 and Figure 1 below. The initial inoculum size for each of these organisms were: GABL (2.7 X 10^3 CFU/ml) and *P. aeruginosa* (1.4 X 10^5 CFU/ml). GABL cell numbers had decreased below the detection limits after 24hrs, and *P. areuginosa* cells had decreased to 74 X 10^2 C FU/ml. No viable cells for either organism was detected after 72 hrs. These data suggest that neither GABL nor *P. aeruginosa* have

the ability to survive in a mineral salts medium, supplemented with Polystep B-27. In addition, these data are consistent with growth measurement data reported for GABL (Table 5) and *P. aeruginosa* (Table 9) in our **Second Quarter report 5/8/02.**

Table 1. Growth Response of GABL and *P. aeruginosa* in Basic Salts Medium (90%), supplemented with Polystep B27 (10%), and incubated at 37° C.

Date	Time (days)	GABL (cfu/ml)	*P. aeruginosa* (cfu/ml)
7/22/02	0	2720	145920
7/23/02	1	0	740
7/24/02	2	0	0
7/25/02	3	0	0

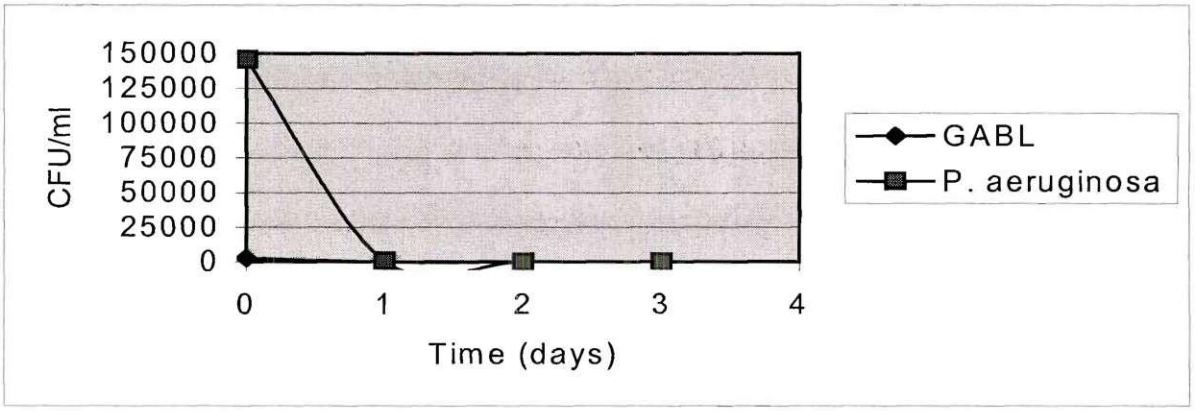

Figure 1. Growth curve for GABL and *P. aeruginosa* in a mineral salts medium with Polystep B27.

Note: The computer program (Microsoft Excel) does not recognize zeroes in logarithmic scale. Therefore, the data is not plotted using logs.

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In our Third Quarter Report (Second-Year Grant period), GABL was evaluated for growth using four variables: (i) inoculated into concentrated emulsion (100% Airflex 400) and incubated at room temperature, (ii) inoculated into concentrated emulsion (100% Airflex 400) and incubated at 37^0C, (iii) inoculated into diluted emulsion (75% Airflex 400 + 25% sterile water), and incubated at room temperature, and (iv) inoculated into diluted emulsion (75% Airflex 400 + 25% sterile water), and incubated at 37^0C. *C. tropicalis* was not evaluated for growth under those conditions. Consequently, for comparative purposes, *C. tropicalis* was evaluated using those four variables during this Fourth Quarter research period, and these data are shown in Table 2. No viable cells were detected in cultures after 24 hrs, or in other samples evaluated periodically, using any of those variables. These experiments were discontinued after four days.

Table 2. Growth Response of *Candida tropicalis* in Airflex 400 (A139).

Date	Time (days)	100% Room (cfu/ml)	100% 37°C (cfu/ml)	75% Room (cfu/ml)	75% 37°C (cfu/ml)
7/29/02	0	3.6×10^3	8.5×10^3	1.25×10^4	1.85×10^4
7/30/02	1	0	0	0	0
8/2/02	4	0	0	0	0

In contrast to data shown for GABL and *P. aeruginosa* in Table 1 and Figure 1, *C. tropicalis* grew well when inoculated into a Basic Salts Medium, supplemented with Polystep B-27 (Table 3, and Figure 2). The initial inoculum was 1.4×10^4 CFU/ml, and cell numbers increased continuously for five days to 6.5×10^6 CFU/ml.

Table 3. Growth Response of *C. tropicalis* in a Basic Salts Medium (90%), supplemented with PolystepB27 (10%) and incubated at 37°C.

Date	Time (days)	*C. tropicalis* (cfu/ml)
7/31/02	0	1.40×10^4
8/1/02	1	1.22×10^5
8/2/02	2	1.64×10^5
8/5/02	5	6.50×10^6

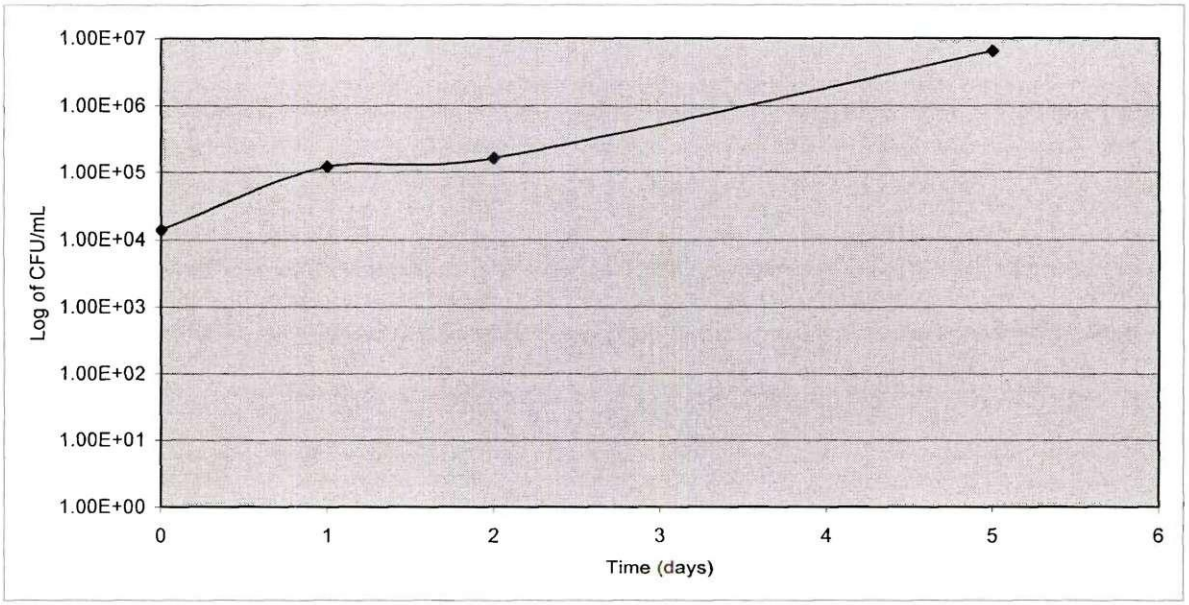

Figure 2. Growth response of *C. tropicalis* in a mineral salts medium, supplemented with PolystepB27, and incubated at 37°C.

In another experiment, we inoculated GABL and *P. aeruginosa* into the Mineral Salts Medium (90%) supplemented with an emulsion component Colloid 675. The initial inoculum was approximately 1.0 X 10^5 CFU/ml for each of the test organisms. After 24 hrs, no viable cells were recovered. We continued to measure growth at 24-hr intervals for seven days, and no viable cells were detected during the sampling period for each of these organisms.

Data in Table 5 and Figure 3 show that GABL grew in the mineral salts medium supplement with the emulsion component Airvol 523, increasing from an initial inoculum of 1.3 X 10^3 CFU/ml to a maximum 5.0 X 10^6 CFU/ml in three days.

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Table 5. Growth Response of GABL in a Mineral Salts Medium Supplemented with the Emulsion Component Airvol 523 and incubated at 37°C.

Date	Time (days)	(cfu/ml)
9/9/02	0	1.3×10^3
9/10/02	1	3.0×10^4
9/12/02	3	5.0×10^6
9/13/02	4	0

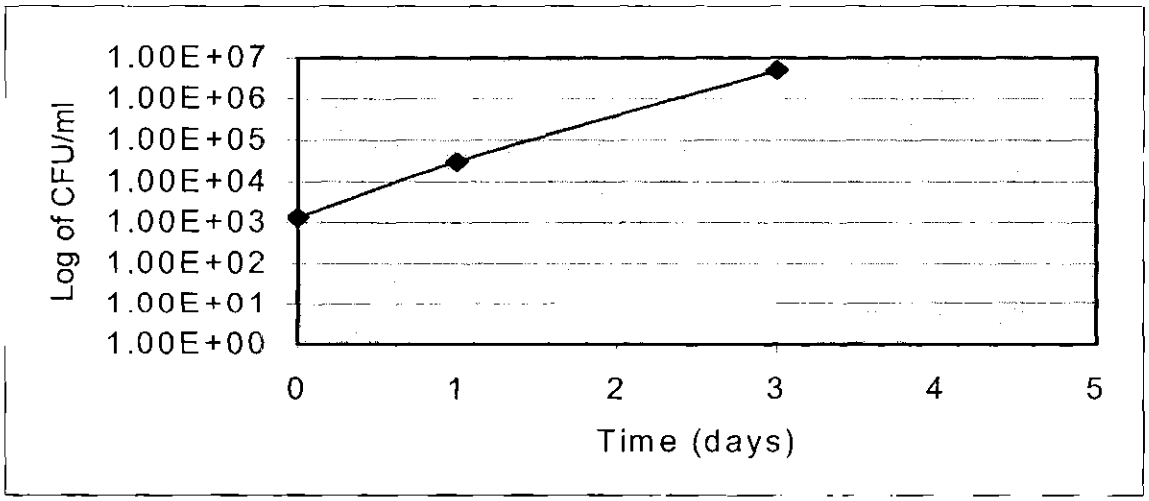

Figure 3. Growth Response of GABL in a Mineral Salts Solution (90%) plus Arivol 523 (10%).

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C. Results of On-going Experiments Designed to Evaluate the nature of Inhibitory Substances Excreted from Cells of GABL and *Pseudomonas aeruginosa.*

We performed additional experiments on the excretory substance produced by *P. aeruginosa* in an effort to demonstrate the presence of lytic phages and/or bacteriocins. First, cell-free extract collected from **cells in the "stationary phase" of growth** was diluted through a series of two-fold dilutions. a) one drop from each tube in the series of six (two-fold) dilutions was tested for activities of "lytic phages" (4,5,10) that would form characteristics **plaques** on a lawn (i.e., confluent growth on the surface of an agar plate) of *P. aeruginosa* and of GABL. b) Then, a second drop from the same tubes were added to a lawn of each organism, and observed for "lysis" (halos) that would be indicative of inhibition by bacteriocins or killer protein (2,4). Results from those experiments were negative.

In separate experiments, we used cell-free extracts from **cells in the "exponential phase" of growth**, and prepared a series of two-fold dilutions as described above. The rationale for these experiments to perform "rapid-test" that may suggest the presence of a **"quorum-sensing molecule"** (regulated by a density-dependent gene) -- a phenomenon that has been demonstrated to mediate many biological processes in a variety of organisms (1,9) Results from these experiments were not indicative of inhibition that is characteristic of either a "lytic phage", bacteriocin, or killer protein.

V. INTERPRETATION AND SIGNIFICANCE

I) Data from growth measurements. Our results confirmed that neither *P. aeruginosa* nor GABL grew in a Mineral Salts Medium, supplemented with the emulsion component Polystep B-27. Our specific experiments were conducted over a relative short-time. These findings are not definitive, and we should consider other factors that may account for the growth response of these organisms, such as length of exposure to a compount and cell density of the culture. Consequently, over a longer exposure time viable organisms may adjust their metabolic machinery, and growth may occur. It is also important to recognize that **"Quorum Sensing"** is a phenomenon that must be considered when interpreting these data. Quorum sensing is defined as regulatory mechanisms used by many different species of gram negative bacteria to control gene expression in response to population density (1,3,9). One group of quorum-sensing regulatory indicator molecules are acyl-homoserine-lactones that control many different activities, such as bioluminescence, in *Vibrio fisheri* (1) antibiotic synthesis (9), and biofilm formation.

Data from growth measurements: *C. tropicalis* (a yeast) grew well in a Mineral Salts Medium supplemented with Polystep B-27, and GABL grew in a Mineral Salts medium supplemented with Airvol 523.

II) Studies on inhibitory substances. Bacteriophages are common to all species of bacteria, but due to their low concentration in the environment, they must be propagated in host strains. Bacteriocins and killer toxins are difficult to demonstrate, because genes that code for their synthesis are regulated by many factors (2,3,6,8).

VI. CONCLUSIONS AND COMMENDATIONS

Research conducted during the past two years have generated experimental data show that many organisms isolated from contaminated emulsions behave as **"dominant species"** and secret **"inhibitory substances"** that prvent growth of other species.

In an effort to demonstrate **"biodegradation activity"**, we (in consultations with Dr. John Rabasco) narrowed the list of species for extensive study to organisms that had been isolated from contaminated emulsion with a relatively high frequency: *Gluconoacetobacter liquefaciens* (GABL), *Pseudomonas aeruginosa*, and *Candida tropicalis*. Each of these species were tested for its ability to grow (using several variables): (a) **in single polymer emulsions** (Airflex 400, before biocide), and (Airflex 192, before biocide), and (b) **in a Mineral Salt Medium (90%) supplemented with single emulsion components (10%).** The emulsion components were: Aerosol 102; Polystep B27; Airvol, 205; Igepal CO-887; Airvol 523; and Colloid 675). Our rationale for these experiments was that the ability to grow (increase in cell numbers) in a pure polymer emulsion, or in a mineral salts medium, supplemented with an emulsion component is a **"prerequisite"** for presumptive biodegradation activity.

We also performed some specialized experiments with **"cell-free" extracts** from GABL and *Pseudomonas aeruginosa* in an attempt to generate data that would held to explain whether **inhibitory activity** was resulting from **"bacteriophages", "bacteriocins", or "killer polypeptides".** Results from these experiments were not definitive. I propose to continue this work (using more specialized methods) in an effort to generate some definitive information and will share results with Air Products.

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VIII. APPENDIX (Dr. F. J. Schork's Polymer Analyses)

Microbiology of Polymer Emulsions

Sponsored by

Air Products and Chemicals

Summary of Polymer Analysis

F. J. Schork
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November 7, 2002

Latex 1

Airflex 400 with GABL

Time (week)	Date	PH	Viscosity (cp)	Conductivity (μs)	Insoluble fraction	325 mesh grit (ppm)	Radius (nm)
Original latex		4	2300	1502	0.66	10	287
0	8/22/01	4	2050	1503	0.63	15	264
1	8/29/01	4	1900	1500	0.64	30	237
2	9/5/01	4	2350	1468	0.65	20	216
3	9/12/01	4	2300	1421	0.65	80	247
4	9/19/01	4	2750	1350	0.64	100	261
5	9/26/01	4	2800	1281	0.64	150	263
6	10/3/01	4	3000	1178	0.65	210	267
7	10/10/01	4	3800	1079	0.65	250	274

Notes:

- Viscosity: Brookfield viscometer
- Insoluble fraction: Air-product method
- Grit: Air-product method
- Radius: dynamic light scattering, Protein Solutions DynaPro

Notes on Results:

- Viscosity rises with age. Viscosity rise in an emulsion generally associated with smaller particle size (unlikely here) or an increase in viscosity of the continuous phase (water soluble polymer formation?)
- Conductivity is down. This is consistent with smaller particles (unlikely) due to surfactant loss from the aqueous phase. Could the explanation instead be microbial? What is in this latex? Low molecular weight surfactant? Salts?
- Insolubles are constant. No change in gel level. This is expected.
- Grit is up. This might indicate some coagulation/flocculation. This might be inconsistent with the viscosity and conductivity data, but very believable in the context of emulsion polymers.
- Particle size goes up slightly. This is probably a result of coagulation.
- Dynamic Mechanical Analysis (DMA) shows little or no change in the mechanical properties of the polymer.
- Molecular weights were not done because the particle and aqueous phases could not be separated.

Latex 2

Airflex 400 with GABL

Time (week)	Date	PH	Viscosity, cp	Conductivity, uS	Grit, ppm	Toluene Insoluble Fraction	Particle Radius nm
Before inoculation	11/01/01	3	3000	1305	10	0.68	338
After inoculation	11/01/01	3	1900	1630	8	0.66	
1	11/07/01	3	1800	1508	8	0.60	280
2	11/14/01	4	1800	1534	20	0.59	270
3	11/22/01	4	2150	1505	66	0.63	290
4	11/28/01	4	2350	1500	76	0.62	285
5	12/05/01	4	2950	1485	175	0.62	280
6	12/12/01	4	3300	1498	90	0.61	
12*	1/23/01	4.5	793	1500	40000	0.65	260

Notes:

- (*) Indicates new technicians
- No final analyses DMA, etc. done due to lack of sample
- Particle size is for the major peak. There is a secondary peak containing approximately 20% of the mass with a radius of approximately 10-40 microns. This could be dust (not likely), microbes, or coagulum. Dilute suspensions were sonicated for 20 minutes before particle size measurement so whatever the composition of the large particle size peak, it cannot be de-agglomerated. Also, one should note that the particle size instrument is NOT accurate at such large sizes.
- Ran out of material before final analyses were done.

Latex 3

Airflex 192 with GABL

Time (week)	Date	PH	Viscosity, cp	Conductivity, uS	Grit, ppm	Toluene insoluble fraction	Particle Radius nm
Before inoculation	11/01/01	4	124	3260	4	0.62	
After inoculation	11/01/01	4	116	3605	15	0.62	
1	11/07/01	4	120	3480	10	0.64	180
2	11/14/01	4.5	124	3430	8	0.63	200
3	11/22/01	4.5	140	3450	26	0.61	150
4	11/28/01	4.5	144	3500	44	0.59	
5	12/05/01	4.5	192	3450	111	0.57	185
6	12/12/01	4.5	212	3360	29	0.61	
12*	1/23/02	4.5	346	3400	130	0.81	150

Notes:

- (*) Indicates new technicians and new spindle/rpm for viscosity.
- No final analyses DMA, etc. done due to lack of sample
- Particle size is for the major peak. There is a secondary peak containing approximately 20% of the mass with a radius of approximately 10-40 microns. This could be dust (not likely), microbes, or coagulum. Dilute suspensions were sonicated for 20 minutes before particle size measurement so whatever the composition of the large particle size peak, it cannot be de-agglomerated. Also, one should note that the particle size instrument is NOT accurate at such large sizes.
- Ran out of material before final analyses were done.

Latex 4

Airflex 400 with GABL

Week	Date	pH	Viscosity (cp)	Conductivity (μS)	Grit (%)	Gel Content (%)	Particle Radius (nm)
1	2/4/02	4.5	800	1500	0.09	76.9	292
2	2/11/02	4.5	880	1400	0.15	35.5	248
3	2/19/02	4.5	870	1800	0.08	38.0	252
5	2/25/02	4.5	840	1600	0.06	37.8	247

Notes:

- Particle size is for the major peak. There is a secondary peak containing approximately 20% of the mass with a radius of approximately 10-40 microns. This could be dust (not likely), microbes, or coagulum. Dilute suspensions were sonicated for 20 minutes before particle size measurement so whatever the composition of the large particle size peak, it cannot be de-agglomerated. Also, one should note that the particle size instrument is NOT accurate at such large sizes.
- Large final sample and large final control not received.

Latex 5

Airflex 192 with GABL

Week	Date	pH	Viscosity (cp)	Conductivity (μS)	Grit (%)	Gel Content (%)	Particle Radius (nm)
1	2/14/02	4.5	100	3400	0.045	72.8	159
2	2/11/02	4.5	100	3500	0.026	37.4	149
3	2/18/02	4.5	100	3700	0.007	42.0	140
4	2/25/02	4.5	110	3500	0.010	42.1	143

Notes:

- Particle size is for the major peak. There is a secondary peak containing approximately 20% of the mass with a radius of approximately 10-40 microns. This could be dust (not likely), microbes, or coagulum. Dilute suspensions were sonicated for 20 minutes before particle size measurement so whatever the composition of the large particle size peak, it cannot be de-agglomerated. Also, one should note that the particle size instrument is NOT accurate at such large sizes.
- Large final sample and large final control not received.

Latex 6

Airflex 400 with Candida

Week	Date	pH	Viscosity (cp)	Conductivity (μS)	Grit (%)	Gel Content (%)	Particle Radius (nm)
Std. Dev.			11	23			12
1	3/12/02	4	2260	1420	0.044	44.8	240
2	3/19/02	4	2060	1310	0.056	44.1	250
3	3/25/02	4	2250	1360	0.048	45.0	240

Notes:

- Standard deviation for viscosity is based on the same spindle and rotational speed. For non-Newtonian fluids, varying the shear rate will vary the viscosity. For this reason, viscosities should be viewed as relative within the same spindle and rpm.
- Viscosity by Brookfield Viscometer with spindle #6 at 100 RPM.
- No viable organisms after Week 3.

Latex 7

Airflex 192 with Candida

Week	Date	pH	Viscosity (cp)	Conductivity (μS)	Grit (%)	Gel Content (%)	Particle Radius (nm)
Std. Dev.			11	19			1
1	3/12/02	4	190	3000	0.014	46.3	150
2	3/19/02	4	180	3000	0.039	47.0	155
3	3/25/02	4	180	3000	0.044	45.1	135
4	4/1/02	4	200	3000	0.018	47.3	150
5	4/9/02	4.0-4.5	190	2930	0.015	46.6	150
6	4/16/02	4.0-4.5	190	2970	0.014	48.4	140
7	4/23/02	4	220	2990	0.024	45.7	135

Notes:

- Standard deviation for viscosity is based on the same spindle and rotational speed. For non-Newtonian fluids, varying the shear rate will vary the viscosity. For this reason, viscosities should be viewed as relative within the same spindle and rpm.
- Viscosity by Brookfield Viscometer with spindle #6 at 100 RPM.

Latex 8

Airflex 400 with GABL

Week	Date	pH	Viscosity (cp)	Conductivity (μS)	Grit (%)	Gel Content (%)	Particle Radius (nm)
Std. Dev.			11	23			12
1	5/28/02	6.0	2480	1500	0.006	72.1	150
2	6/3/02	6.0	2680	1320	0.017	73.2	280
3	6/10/02	5.5	1520	1830	0.017	69.2	320
4	6/17/02	5.5	1280	1830	0.016	63.6	290

Notes:

- Standard deviation for viscosity is based on the same spindle and rotational speed. For non-Newtonian fluids, varying the shear rate will vary the viscosity. For this reason, viscosities should be viewed as relative within the same spindle and rpm.
- Viscosity by Brookfield Viscometer with spindle #6 at 100 RPM.
- Particle size data for Week 1 probably in error.
- No viable organisms after Week 3.

Latex 9

Airflex 192 with GABL

Week	Date	pH	Viscosity (cp)	Conductivity (μS)	Grit (%)	Gel Content (%)	Particle Radius (nm)
Std. Dev.			11	19			1
1	5/28/02	6.0	100	3760	0.044	79.0	162
2	6/3/02	6.0	110	3780	0.008	76.4	220
3	6/10/02	6.0	120	3770	0.045	750.	250
4	6/17/02	6.0	120	3680	0.005	72.6	257

Notes:

- Standard deviation for viscosity is based on the same spindle and rotational speed. For non-Newtonian fluids, varying the shear rate will vary the viscosity. For this reason, viscosities should be viewed as relative within the same spindle and rpm.
- Viscosity by Brookfield Viscometer with spindle #6 at 100 RPM.
- Particle size data for Week 1 may be in error.
- Particle size goes up slightly. This is probably a result of coagulation. No corroboration in the conductivity. (Coagulation should increase the conductivity.)

Final and Control Analyses

Sample	pH	Conductivity (µS)	Viscosity cp	Gel Content % by mass gel	Grit Test % by mass grit	Particle Size (nm)	Molecular Weight Mn	Mw	MW Poly-dispersity
Airflex 400 Candida (Latex 6)	5.0	1.52	407.0	70.09	0.077	259.25	132,100	216,300	1.637
Airflex 192 Candida (Latex 7)	6.0	3.13	310.0	76.20	0.008	187.35	78,590	175,100	2.228
Airflex 400 GABL (Latex 8)	6.0	1.84	161.0	57.65	0.030	269.75	110,700	236,000	2.132
Airflex 192 GABL (Latex 9)	6.0	3.76	160.0	72.65	0.024	168.64	111,900	200,100	1.788
Airflex 400 Control	5.5	1.41	265.0	69.35	0.021	269.06	107,800	196,900	1.827
Airflex 192 Control	6.5	3.11	220.0	76.28	0.003	162.64	77,740	165,500	2.129

Notes:

- All analyses (except dynamic mechanical analyses) performed on 7/30/02
- Viscosity by Brookfield Viscometer with spindle #6 at 100 RPM.
- Molecular weight by gel permeation chromatography on the soluble fraction of the latex.
- MW Polydispersity is the polydispersity of the molecular weight distribution.
- Dynamic Mechanical Analyses are attached.
- NMR was not possible because the samples contained a large fraction of gel.
- FTIR was done, but was inconclusive due to low signal to noise ratio (due to thick films).
- Significant drop in viscosity for Airflex 400 with GABL and Candida relative to control. Some reduction in molecular weight as well.

Overall Notes:

- A significant error could result with grit measurement because of the limited sample weight (one order of magnitude less than the recommendation from Air Products). Grit was done by 100 Mesh screen.
- Viscosity rises with age for Latex 1. Viscosity rise in an emulsion generally associated with smaller particle size (unlikely here) or an increase in viscosity of the continuous phase (water soluble polymer formation?)
- Significant drop in viscosity for Airflex 400 with GABL and Candida relative to control. Some reduction in molecular weight as well.
- Particle size goes up slightly for Latex 9. This is probably a result of coagulation. No corroboration in the conductivity. (Coagulation should increase the conductivity.)
- Some increase in modulus (E' at low frequency asymptote) for Airflex 192 treated with Candida and GABL over the control.
- No glass transitions visible in any of the samples (by dynamic mechanical analysis).

Dynamic Mechanical Spectra
(October 10, 2001)
Latex 1 (Airflex 400 with GABL)
Control

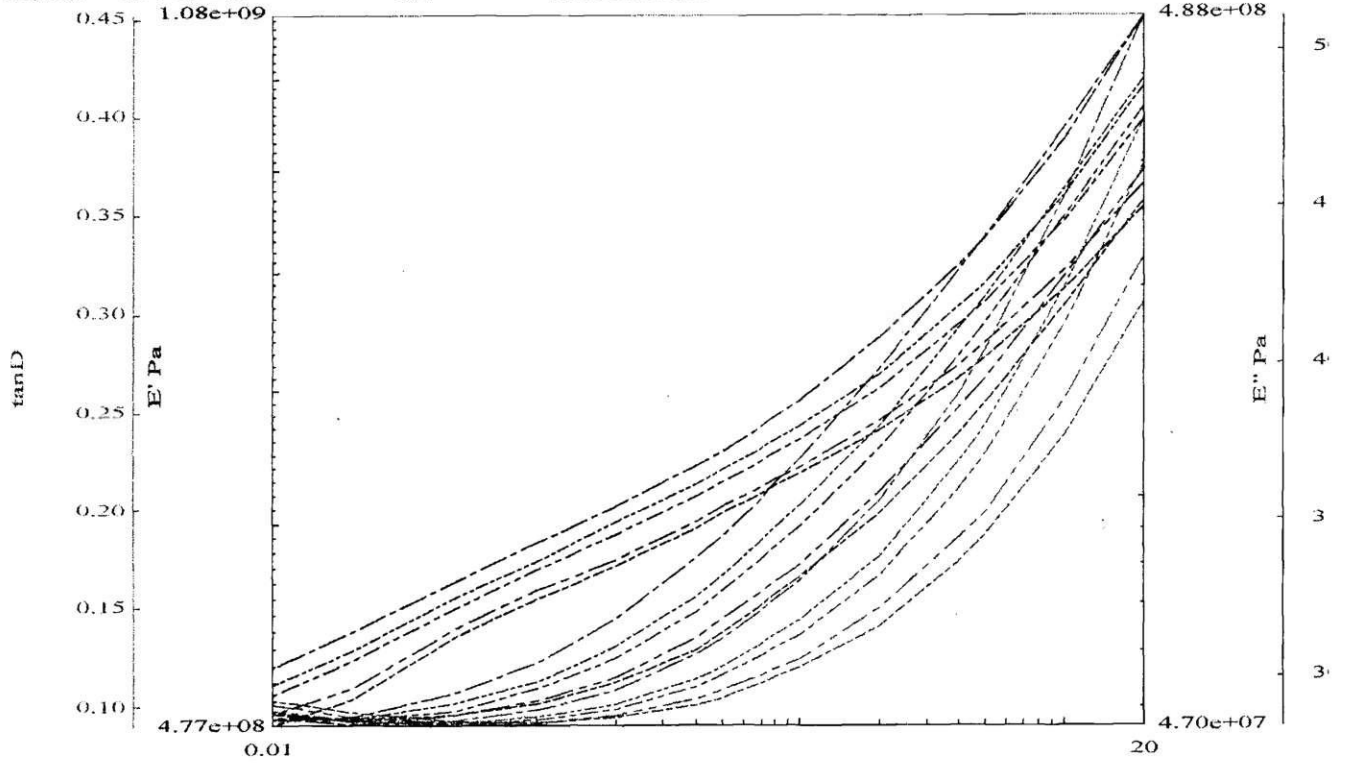
<<< DMS >>>
Data Name: L1
Date: 1/10/21 20:41
Sample: L1
Length*Width*Thickness:
15* 0.25* 0.55 mm
Deformation: Tension
Frequency: 0.01-20 Hz

Temperature Program:
25- 30C 10Steps
Soak: 100 sec

Slice:
25 C
26 C
27 C
28 C
29 C

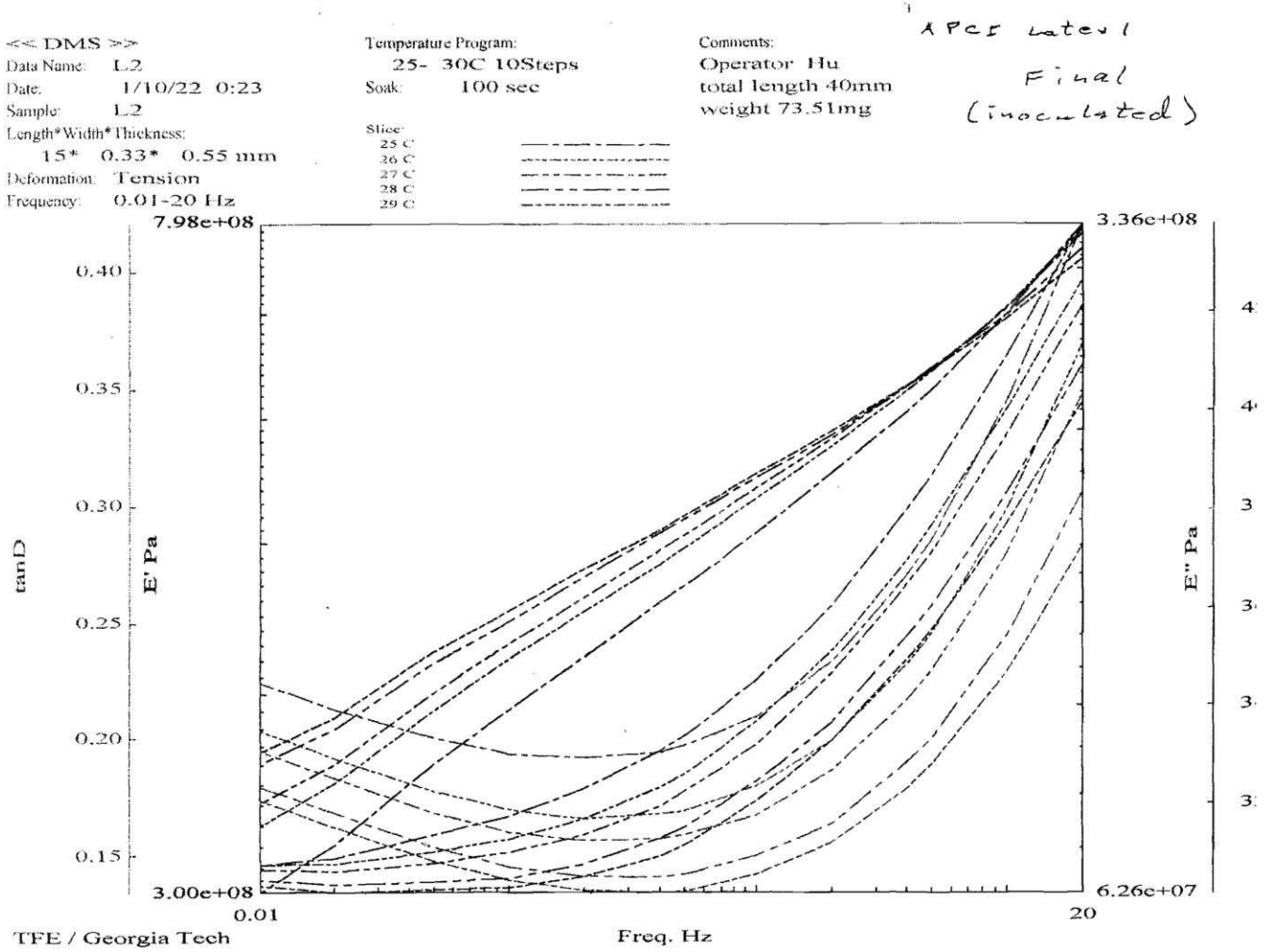
Comments:
Operator Hu
total length 40mm
weight 61.51mg

APCI Latex 1
initial
(control)
not inoculated



TFE / Georgia Tech

Dynamic Mechanical Spectra
(October 10, 2001)
Latex 1 (Airflex 400 with GABL)
Final Inoculated


APCF latex 1
Final
(inoculated)
<<< DMS >>>
Data Name: L2
Date: 1/10/22 0:23
Sample: L2
Length*Width*Thickness:
15* 0.33* 0.55 mm
Deformation: Tension
Frequency: 0.01-20 Hz
Temperature Program:
25- 30C 10Steps
Soak: 100 sec
Comments:
Operator Hu
total length 40mm
weight 73.51mg
Slice:
25 C
26 C
27 C
28 C
29 C
7.98e+08
3.00e+08
3.36e+08
6.26e+07
tanD
E' Pa
E" Pa
0.40
0.35
0.30
0.25
0.20
0.15
0.01
20
Freq. Hz
TFE / Georgia Tech

Dynamic Mechanical Spectra

(October 30, 2002)

Strain controlled dynamic mechanic analysis

Test mode: Dynamic frequency temperature sweep.

Static Tension: 8 g force

Strain: 0.2%

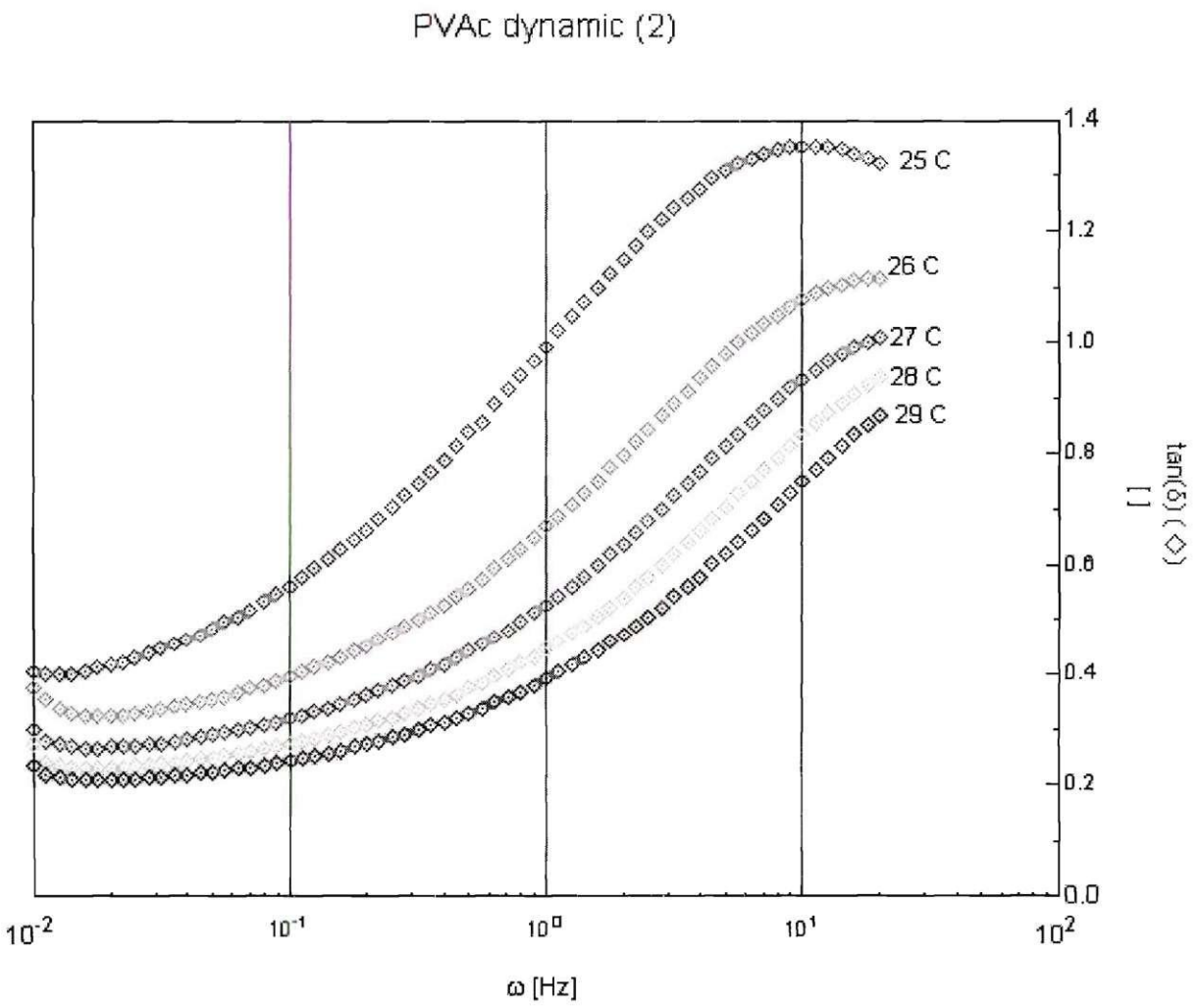
Frequency range: 0.01 to 20 Hz

Temperature: 25 to 29

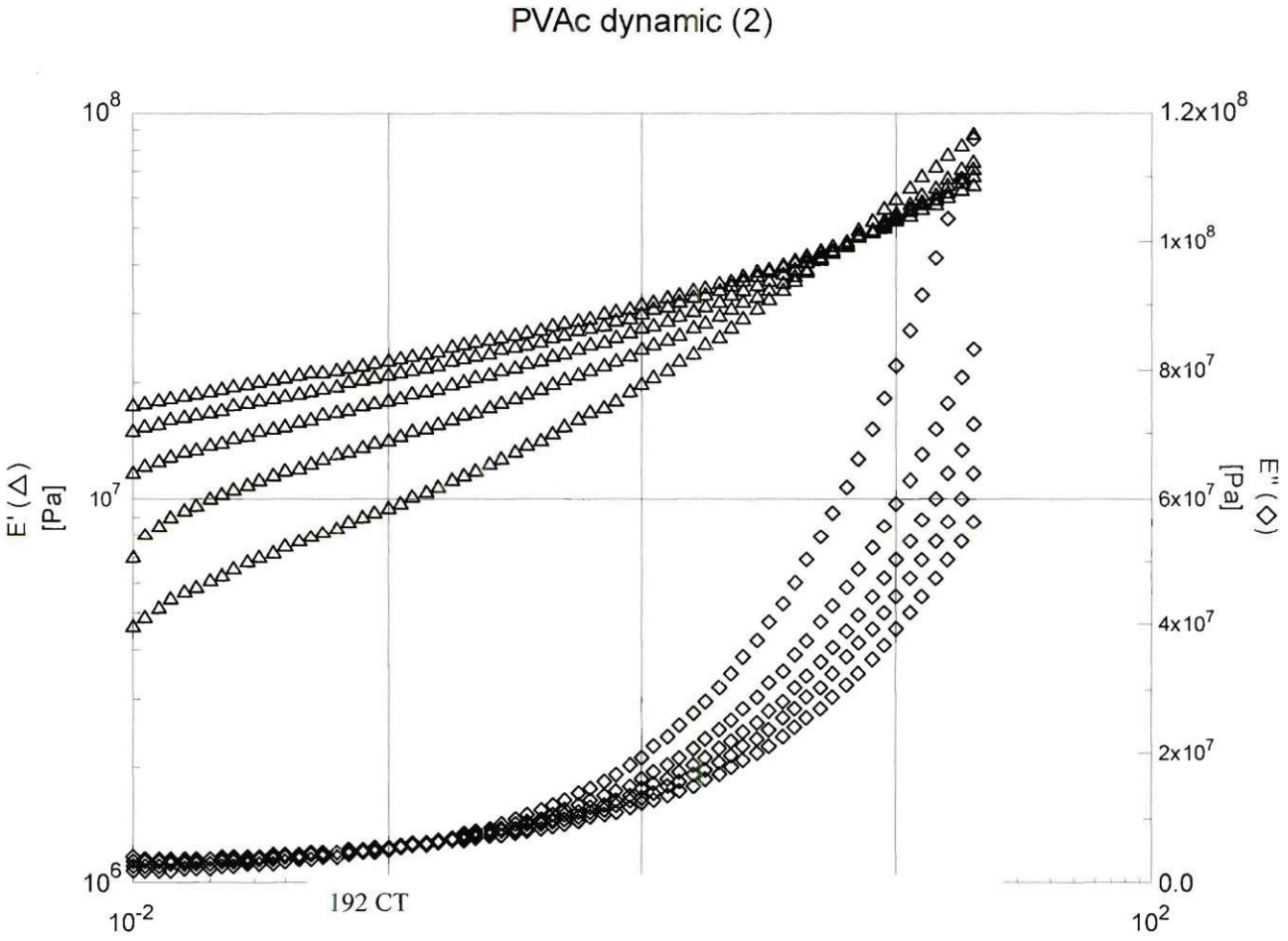
Soak time: 300 sec

Size: 15mm*~1.51mm*~1.68mm

Airflex 192 Candida (Latex 7)

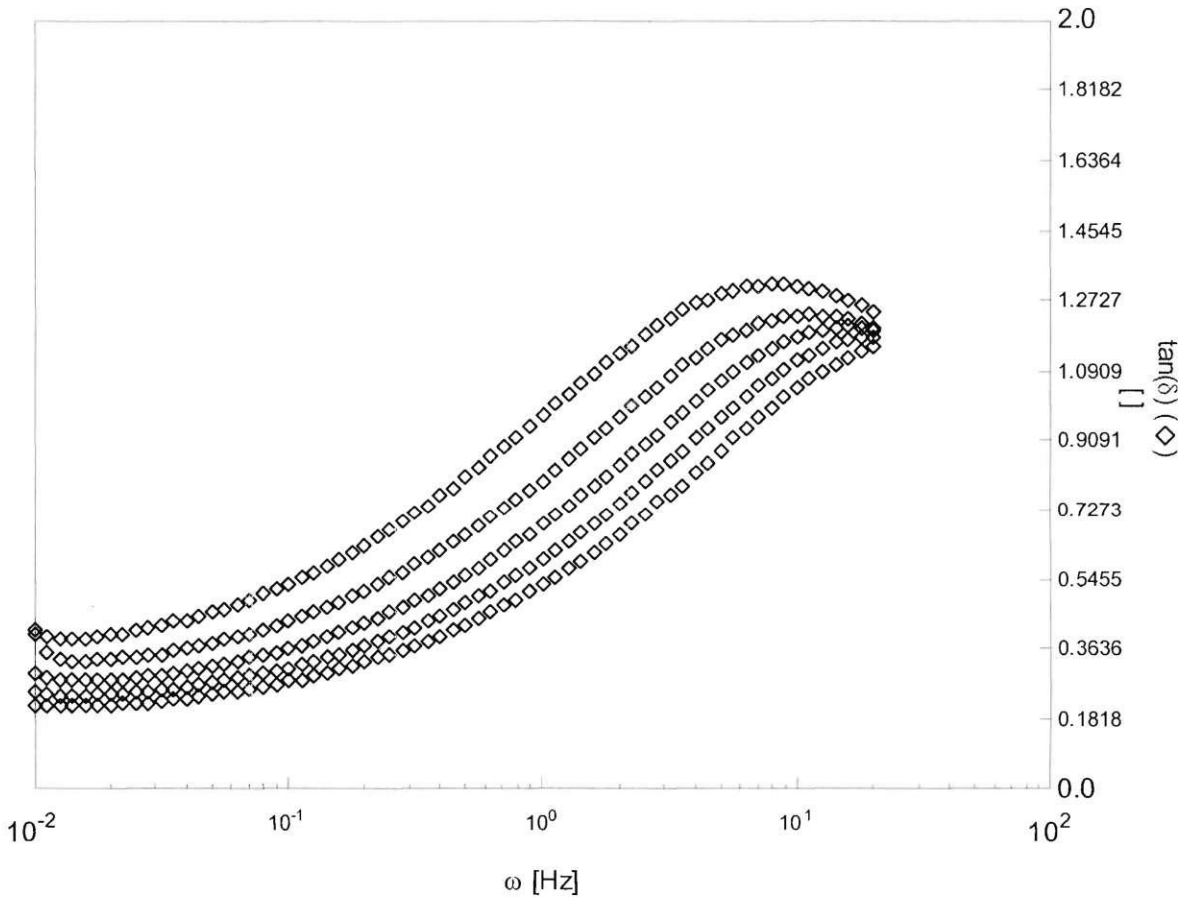


Airflex 192 Candida (Latex 7)



Airflex 192 Control

PVAc 192A35 (4)



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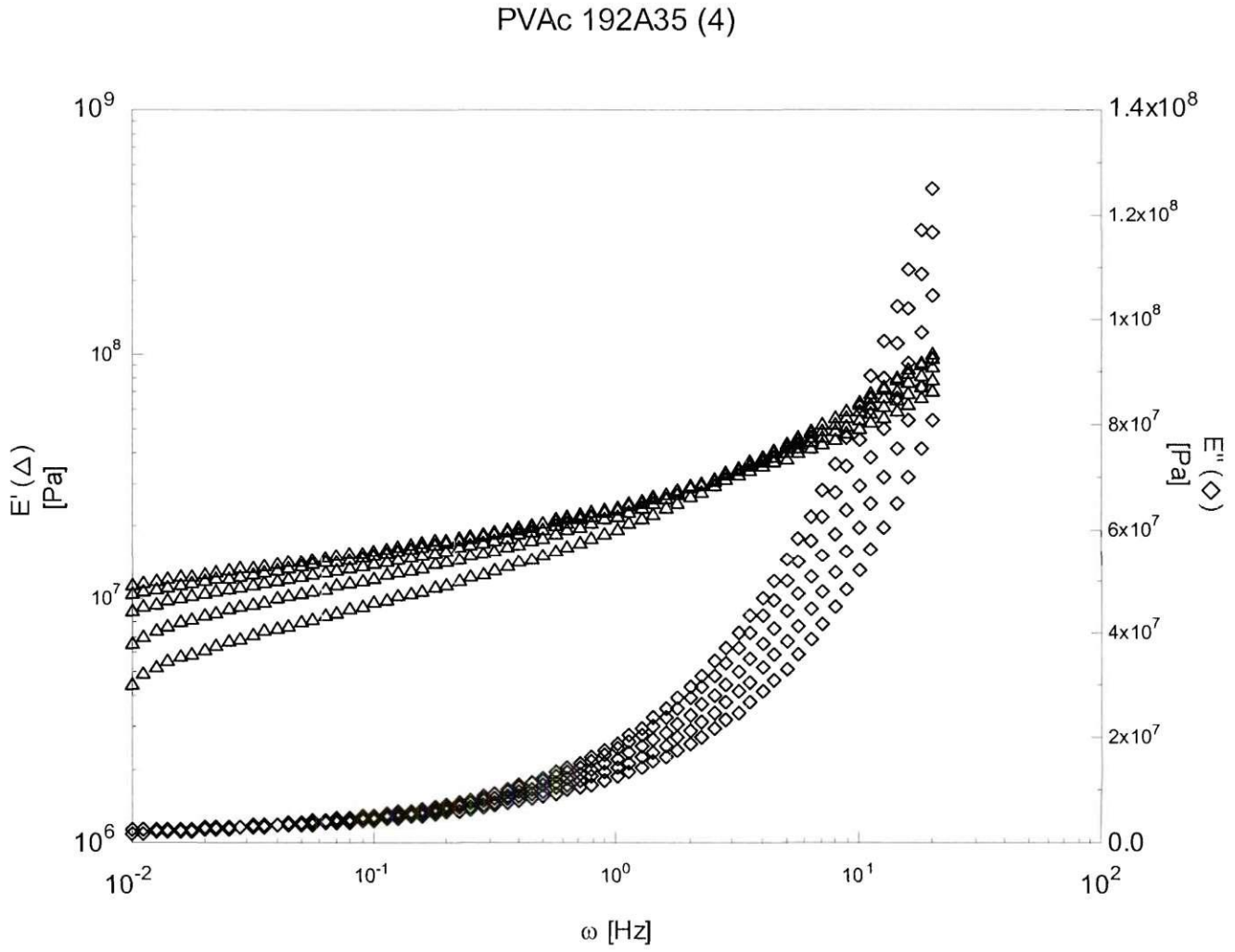
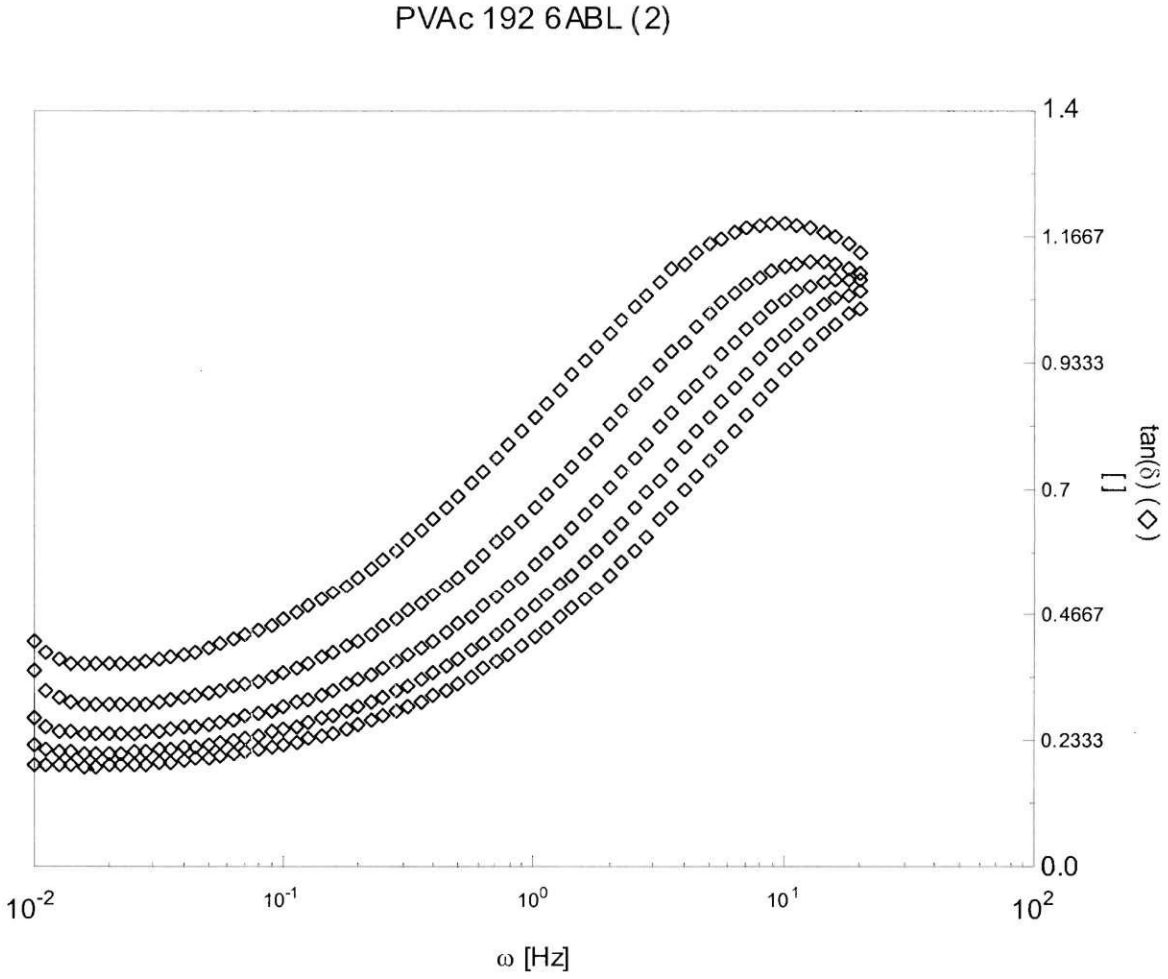
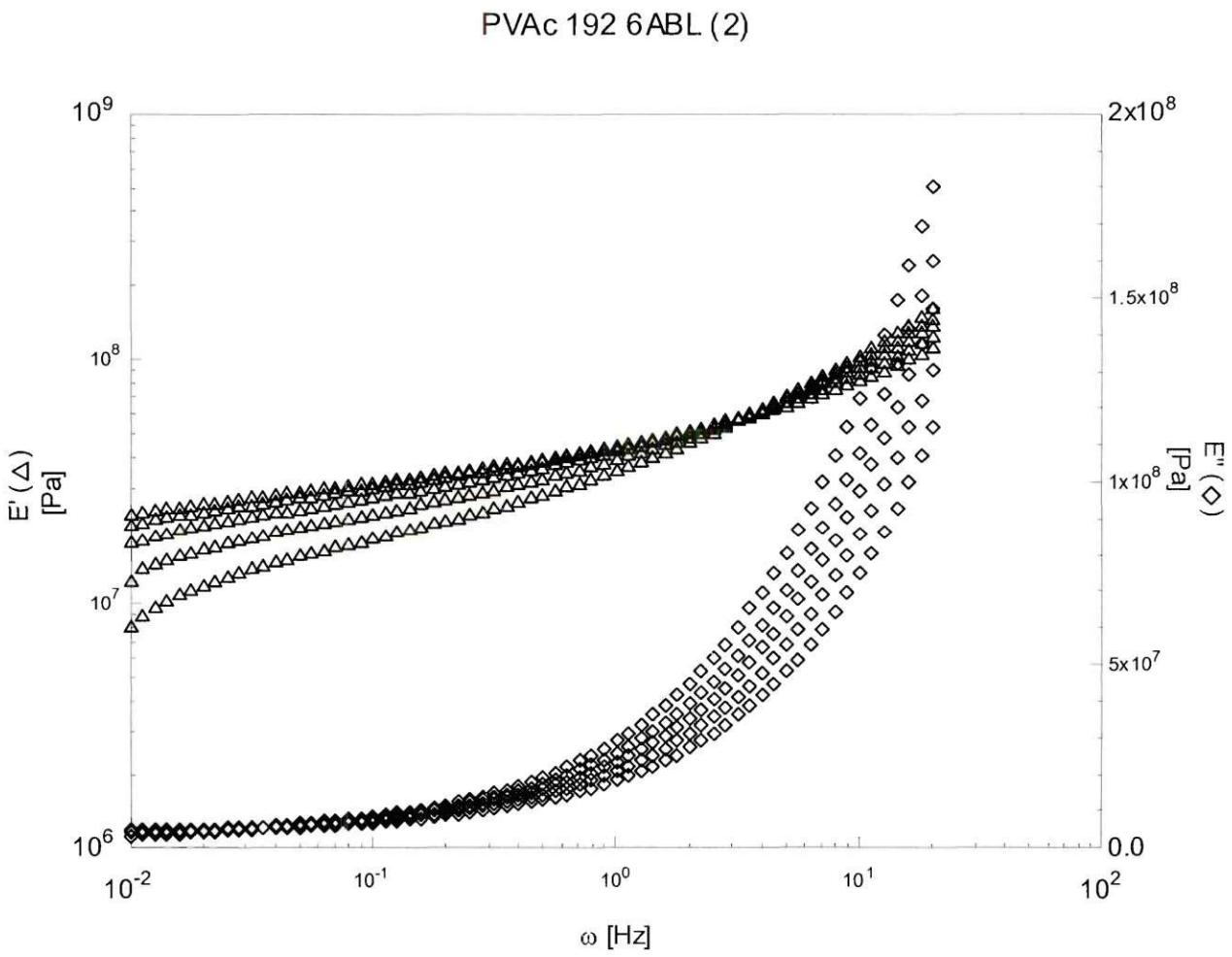


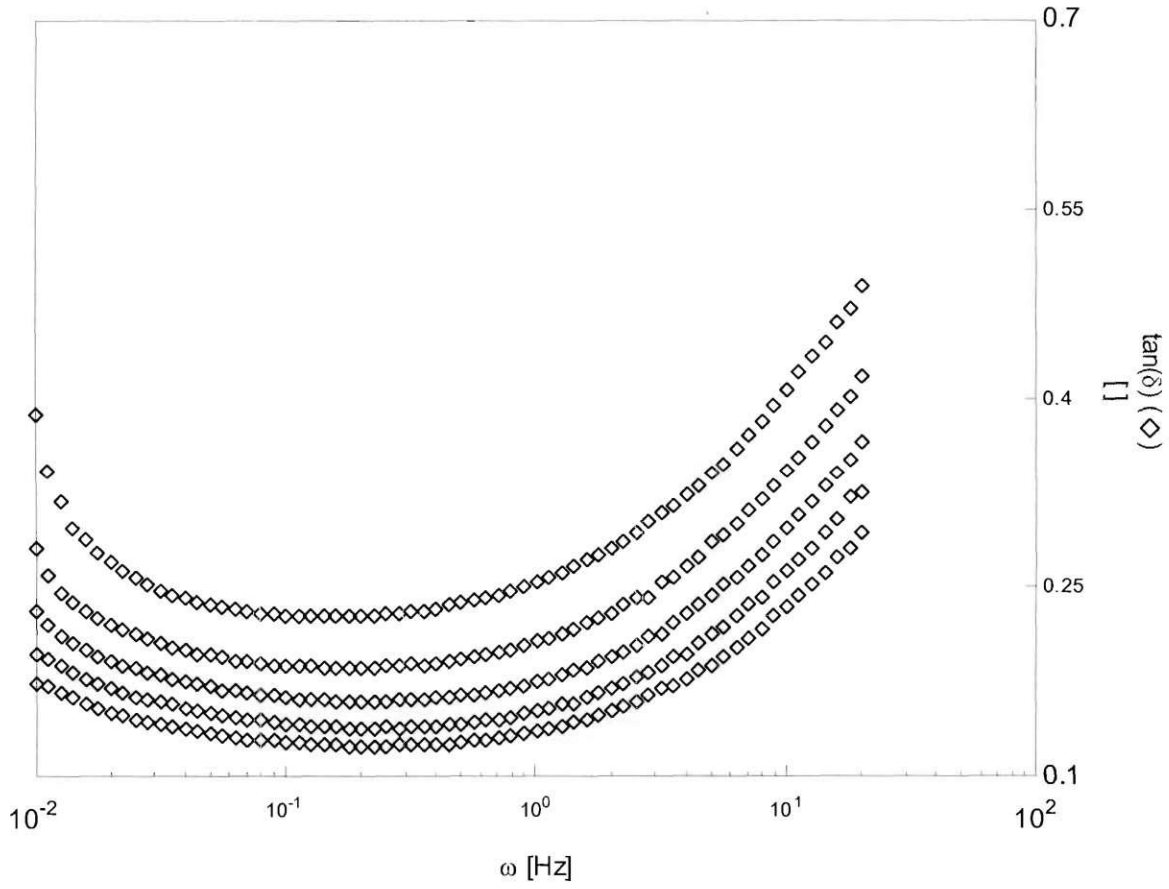
Airflex 192 GABL (Latex 9)



Airflex 192 GABL (Latex 9)



Airflex 400 Candida (Latex 6)



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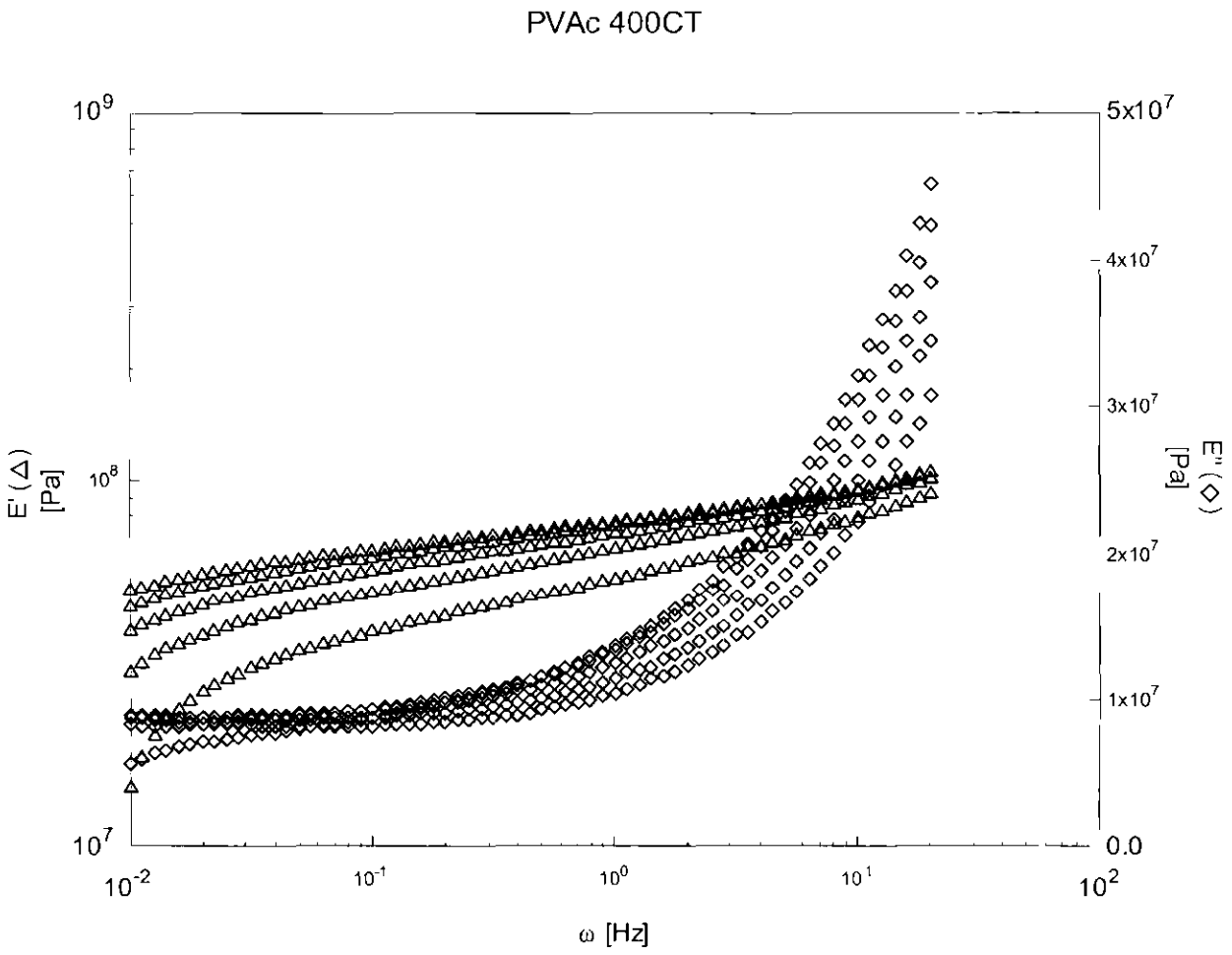
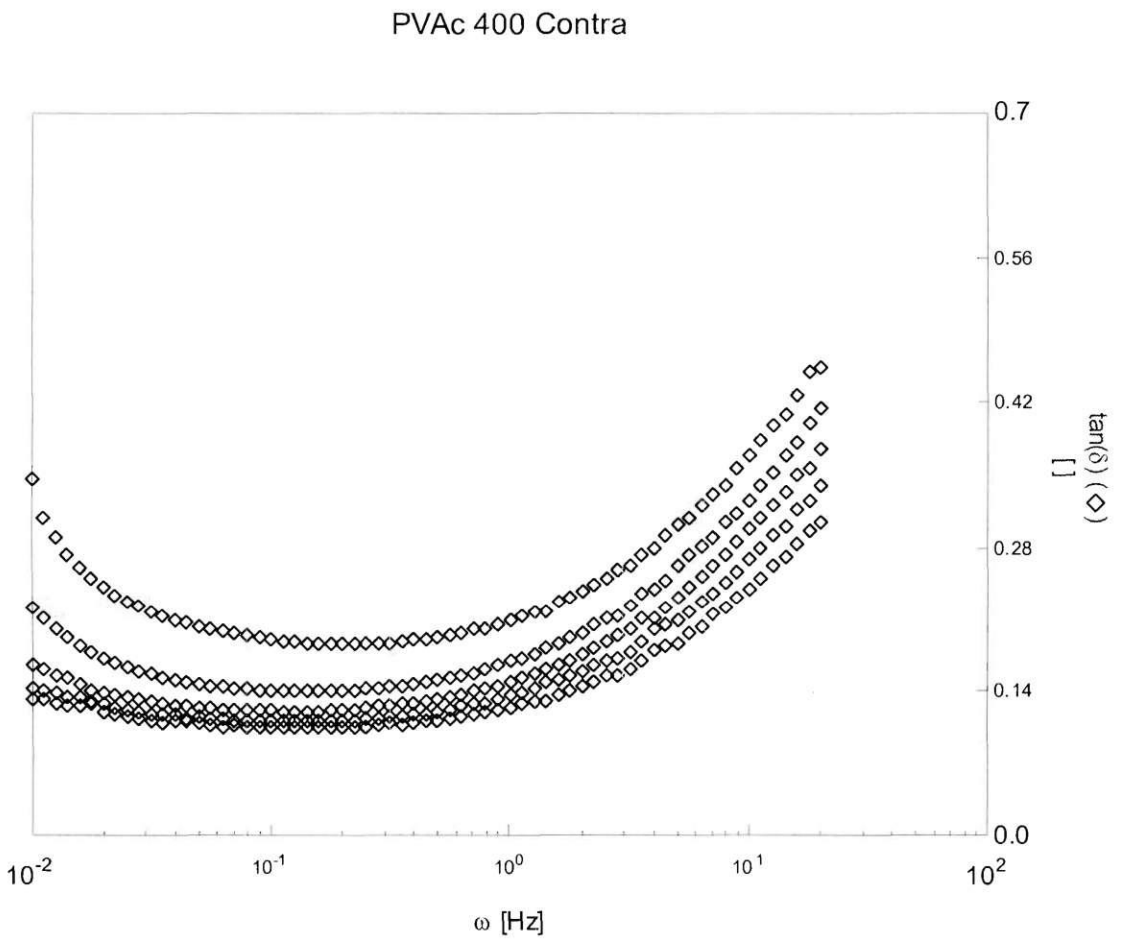
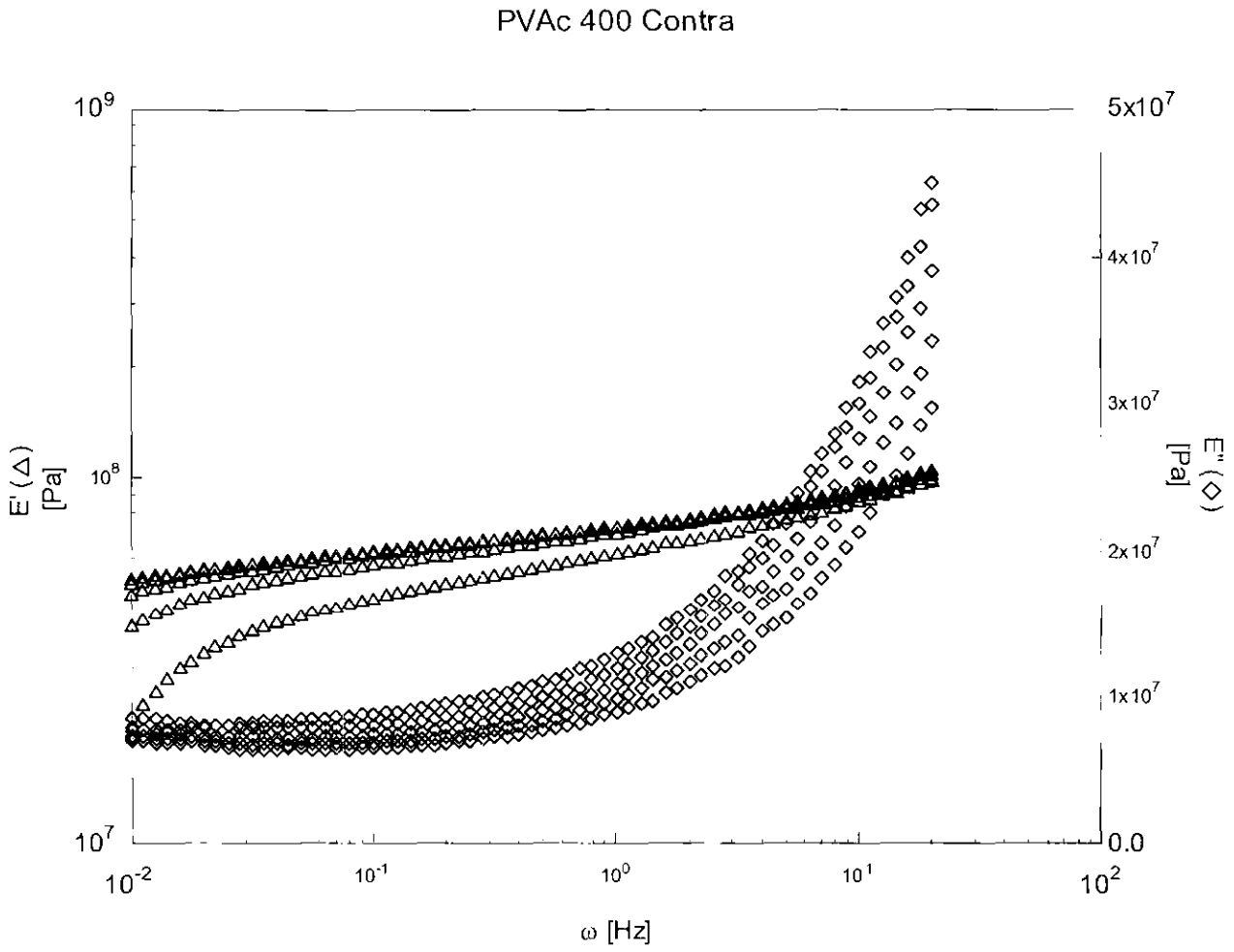


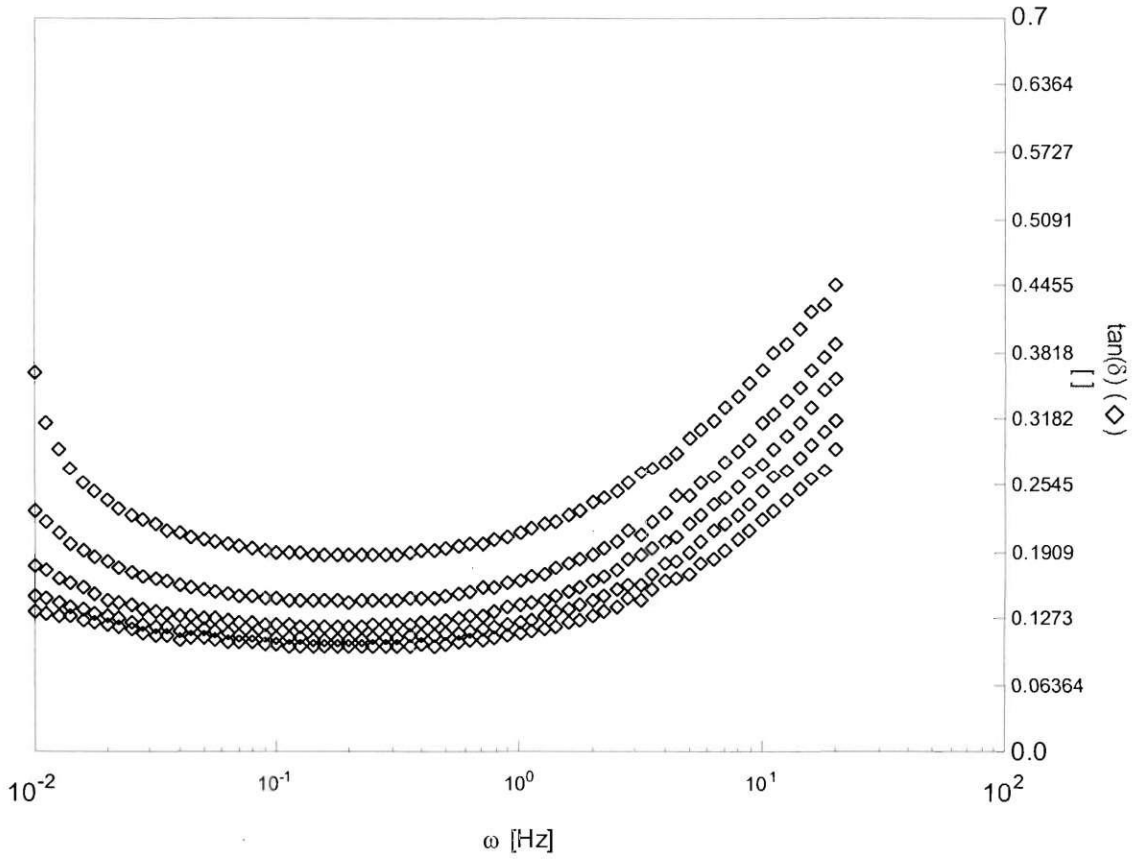
Airflex 400 Control



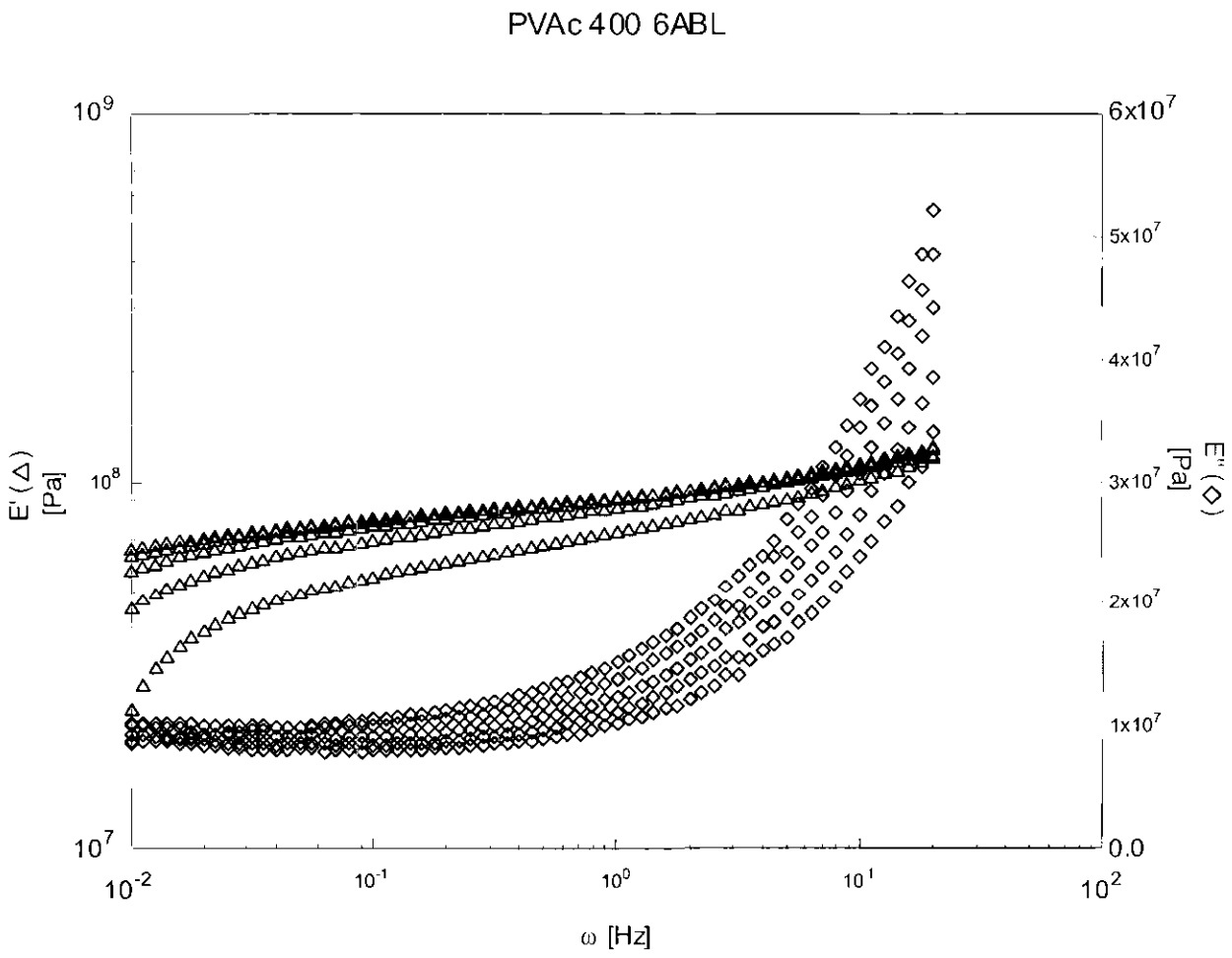
Airflex 400 Control



Airflex 400 GABL (Latex 8)



Airflex 400 GABL (Latex 8)



VI. REFERENCES

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G-32-634

#6

FIRST QUARTER PROGRESS REPORT
(SECOND-YEAR GRANT PERIOD)
September, 2001 - January, 2002

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIONS: BEHAVIOR OF MICROORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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Edmonds, P., Second-Year Grant Period (First Quarter Report, February 15, 2002)

TABLE OF CONTENTS

Edmonds, P., Second-Year Grant Period (First Quarter Report, February 15, 2002)

I. EXECUTIVE SUMMARY

Experimental work outlined and discussed in this report covers an extended time-period (i.e., continuation of experiments begun in the **First-Year of this Grant**), and the **initiation of "new" experiments,** during the First Quarter of the **Second-Year Grant Period**. Collectively, the **major objectives** of these experiments are to generate information that will contribute to our understanding of the biodeterioration processes (chemical and physical changes) in "samples" of Airflex 400 and Airflex 192; each inoculated separately with a pure culture of GABL and a pure culture of *Candida tropicalis*. During these **"continuation experiments",** sub-fractions of each emulsion were removed at pre-determined time periods, and analyzed for growth of the "challenge microorganisms" (in Dr. Edmonds' Laboratory), and a portion of each sample was taken to the School of Chemical Engineering and analyzed for "chemical and physical changes" (in Dr. Joseph Schork's Laboratory). **NOTE: Results from Dr. Schork's Analyses will be forwarded by him separately as an "Addendum" to this report.** Our results of bacterial growth in **continuing experiments** are as follows:

A. Results of GABL's Growth in Airflex 400 (Before Biocide). The initial inoculum of GABL (0-Time Period) was 6.3 X 10^5 CFU/ml. Growth increased to 9.5 X 10^6 CFU/ml, and at the end of four weeks, and the observed **odor change was "Atypical".** No other observed changes were noted through 11 weeks; **when this experiment was terminated due to insufficient volume of this emulsion sample" (500 ml when this experiment was started).** This experiment is being repeated using **1 liter of Airflex 400.**

Edmonds, P. (Second-Year Grant Period), First Quarter Report.

B. Results of GABL's Growth in Airflex 192 (Before Biocide).

Growth of GABL in Airflex 192 increased from an **initial inoculum size of 8.9X10^5 CFU/ml)** to 8.8X10^6 CFU/ml in five weeks, after which the odor change of this emulsion was **"Atypical".** No other qualitative changes were observed at the end of the eleventh week when this experiment was **discontinued, due to insufficient volume of this Emulsion sample (500 ml when the experiment was started).** This experiment is being repeated using **1 liter of Airflex 192.**

C. Results of Studies to Determine Inhibitory Interactions Using a Mixed Culture (GABL + *Pseudomonas aeruginosa*) in Potato Dextrose Broth. This experiment was started using Potato Dextrose Broth (pH 5.0), and a Mixed inoculum (GABL [2.0 X 10^{5}CFU/ml] + *P. aeruginosa*[1.5 X 10^{3}CFU/ml]). GABL increased in seven days to 5.6 X 10^{6}CFU/ml, and *P. aeruginosa* decreased slightly to 8.0 X 10^{2}CFU/ml. Simultaneously, the pH of the medium decreased from pH 5.0 to pH 3.62. These results suggest that the **increase in acidity** of the medium creates an environment that is **unfavorable for growth of** *P. aeruginosa.* In an effort to better interpret these results, another experiment (paragraph below) was performed using GABL's cell-free medium.

D. Results of Mixed Culture Interactions (GABL + *P. aeruginosa*) in Potato Detrose Supernatant (Cell-Free Medium). The mixed inoculum was (GABL [1.5 X 10^{6}CFU/ml] + *P. aeruginosa* [6.8 X 10^{3}CFU/ml]). After 72 hr, GABL's population remained approximately the same 6.3 X 10^{6}CFU/ml, but *P. aeruginosa*'s population decreased to **"zero"** in 24 hr. This is a very dramatic change **(initial pH 4.0)** when

Edmonds, P., Second Year Grant Period (First Quarter Report, 2/15/02

Considering the above **"mixed culture experiment",** where the *P. aeruginosa*'s population decreased only slightly and were viable at pH 3.62, after 7days. **Because these results were unexplainable, two additional "categories" of experiments were conducted and/or planned:**

1) Using cell-free supernatants with pH values adjusted to "neutral" or "acid", prior to inoculation with different organisms, as shown below. Results from those experiments (listed below) suggest that "acidity" of supernatants is inhibiting growth of *P. aeruginosa.*

(a) *P. aeruginosa* inoculated into GABL's cell-free supernatant (pH 3.5 **[No Growth]** and pH 7.0 **[Growth]**).

(b) *P. aeruginosa* inoculated into GABS' cell-free supernatant (pH 3.5 **[No Growth]** and pH 7.0 **[Growth]**).

(c) GABL inoculated into *P. aeruginosa*'s cell-free supernatant (pH 6.7 **[Constant CFU/ml, and No Inhibition]**).

(d) GABS inoculated into *P. aeruginosa*'s cell-free supernatant (pH 6.7**[Constant CFU/ml, and No Inhibition]**).

2) Other Experiments "initiated or planned" are designed to determine if GABL or GABS "excrete" Bacteriocins and/or Lytic Bacteriophages that may "inhibit" the growth of *P. aeruginosa*. These same experiments will be performed using *P. aeruginosa,* because such substances may be responsible for *P. aeruginosa*' s inhibition of GABL and GABS in Cross-Streak experiments (**data included in my 4th Quarter Report, 2001).**

Edmonds, P., Seconds-Year Grant Period (First Quarter Report, 2/15/02).

E. Six on-going experiments (no definitive results as of this date: Evaluation of Growth on Emulsion Components (Aerosol A-102 and Polystep B-27).

Experiment #1. GABL Growth on Aerosol A-102.

Experiment #2. GABL Growth on Polystep B-27.

Experiment #3. *Candida tropicalis* Growth on Aerosol A-102.

Experiment #4. *Candida tropicalis* Growth on Polystep B-27.

Experiment #5. GABS Growth on Aerosol A-102.

Experiment #6. GABS Growth on Polystep B-27.

II. OBJECTIVES OF EXPERIMENTS IN THIS REPORT

A. The **objectives of "growth measurements"** (i.e., challenge experiments) where GABL is inoculated "separately" into **Airflex 400** (Before Biocide) are to generate data that will help to determine if this organism is contributing to **biodeterioration processes** (chemical and physical changes) in this polymer emulsion. **(Note: Physical parameters are measured by Dr. Schork.)**

B. The **objectives of "growth measurements"** (i.e, challenge experiments) where GABL is inoculated "separately" into **Airflex 102 (**Before Biocide) are to generate data that will haelp to determine if this organism is contributing to **biodeterioration processes** (chemical and physical changes) in this polymer emulsion.

C. The **objectives** for **"growth measurements" using a mixed-culture** (GABL+ *P. aeruginosa* in **potato dextrose broth (pH 5.0)** were to determine if **acidity** (generated by GABL) is responsible for the **inhibitory action exhibited** against

Edmonds, P., Second-Year Grant Period, (First Quarter Report, 2/15/02).

P. aeruginosa **(observed in Cross-Streak Experiments, reported in the 4th Quarter Report, 2001).**

D. The **objectives of "growth measurements" of a mixed culture (GABL + *P. aeruginosa*) in Potato Dextrose Broth Supernatant (i.e., cell-free medium, after growth)** to determine if "acidity" in the cell-cell free supernatant is responsible for inhibition the growth of *P. aeruginosa.* **(Note: Several variations of these experiments were conducted, using the "natural" pH value of cell-free supernatants; and a range of artificially adjusted pH values.)**

E. The **objectives of "six on-going experiments"** are to determine if each of three organisms (**GABL, *Candida tropicalis*,** and **GABS),** will metabolize (grow) and/or degrade **emulsion components (Aerosol A-102, and/or Polystep B-27)** when inoculated "separately" into a mineral salts medium "supplemented" with a single emulsion component.

III. METHODS/PROCEDURES/PROTOCOLS

A. Protocols for Growth (interaction experiments) of microorganisms in Emulsions (Before Biocide) are described in the **3rd Quarter Report, 2001**.

B. **Dr. Schrock** will provide "protocols" for chemical and physical analyses performed in his laboratory.

C. Protocols for **"challenge experiments)** to determine if organisms will metabolize (grow) on emulsion components are described in the **2nd Quarter Report, 2001.**

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IV. RESULTS

Results of experimental work described in this report are discussed separately.

Results of GABL growing in Airfles 400 (Before Biocide). The initial inoculum of GABL (0-Hr) was 6.3 X10^5CFU/ml. Growth increased to 9.5 X 10^6CFU/ml, and at the end of 4 weeks the observed **odor change was "Atypical"**. The starting pH 4.5 and thereafter we experience considerable variation due to a faulty electrode. These data are shown in Table 1 (below). This experiment was **terminated due to insufficient volume of emulsion sample (500 ml when the experiment was started),** being repeated with **a larger volume (1 liter).**

Table **1. Results for *GABL* growth on Airflex 400 (before biocide).**

Date/Week	Average cfu/ml	pH	Color change	Odor change	Phase separation
10/31/01, Time 0 before inoculation	0	4.5	None	None	None
10/31/01, Time 0 after inoculation	6.3 x 10^5	4.5	None	None	None
11/7/01, Week 1	5.3 x 10^6	4.7	None	None	None
11/14/01 Week 2	9.5 x 10^6	5.4	None	None	None
11/21/01 Week 3	1.7 x 10^6	?	None	None	None
11/28/01 Week 4	2.4 x 10^6	?	None	+4 = Atypical	None
12/5/01 Week 5	3.0 x 10^6	?	None	+2 = putrid	None
12/12/01 Week 6	3.1 x 10^6	?	None	+2 = putrid	None
1/23/02 Week 11	8.6 x 10^5		None	+2 = putrid	None

? = pH meter was faulty

Results of GABL's Growth in Airflex 192 (Before Biocide). The initial inoculum was 8.9 X 10^5CFU/ml and **increased to** 3.1 X 10^6CFU/ml in six weeks, then

the population showed a decrease 8.6 X 10^5CFU/ml in 11 weeks. These data are shown in **Table 2 (below).** However, this experiment was discontinued due to **insufficient volume (500 ml when the experiment was started).** This experiment is being repeated with a larger volume **(1 liter).**

Table 2. Results for *GABL* growth on Airflex 192 (before biocide)

Date/Week	Average cfu/ml	pH	Color change	Odor change	Phase separation
10/31/01, Time 0 before inoculation	0	4.6	None	None	None
10/31/01, Time 0 after inoculation	8.9×10^5	4.6	None	None	None
11/7/01, Week 1	1.2×10^4	4.8	None	None	None
11/14/01 Week 2	3.0×10^5	5.2	None	None	None
11/21/01 Week 3	1.1×10^6	?	None	None	None
11/28/01 Week 4	4.3×10^6	?	None	None	None
12/5/01 Week 5	8.8×10^6	?	None	+4 = Atypical	None
12/12/01 Week 6	3.4×10^6	?	None	+4 = Atypical	None
1/23/02 Week 11	3.8×10^5	?	None	+4 = Atypical	None

? = pH meter was faulty

GABL appeared to have grown in the two emulsions up to week 6 then the cell count dropped by week 11. There is also a change in odor with both emulsions. There is however no color and separation change. The results indicated by the Airflex 400 study are consistent with the previous study included in the 3rd and 4th quarter results. There is however no phase separation due to the mixing of the emulsions prior to taking samples. **(Note: Mixing was recommended by Dr. Schork for his analyses).**

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Table 3. Results for *GABL* Mixed With *Pseudomonas aeruginosa* in Potato Dextrose Broth (adjusted to pH 5.0)

Time	*GABL* plated on potato dextrose agar (cfu/ml)	*P. aeruginosa* plated on cetrimide agar (cfu/ml)	pH
0 hr	2.0×10^5	1.5×10^3	5.0
24 hr	1.41×10^6	2.39×10^3	4.91
48 hr	6.1×10^6	3.0×10^3	4.74
72 hr	9.0×10^6	3.3×10^3	4.12
4 days	7.2×10^6	3.3×10^2	4.0
5 days	7.6×10^6	3.7×10^2	3.68
6 days	6.5×10^6	1.81×10^3	3.67
7 days	5.6×10^6	8.0×10^2	3.62

Table 4. Results for *GABL* Mixed With *Pseudomonas aeruginosa* grown in *GABL* supernatant (pH adjusted 4.0)

Date/Time	*GABL* plated on potato dextrose agar (cfu/ml)	*P.aeruginosa* plated on cetrimide agar (cfu/ml)	pH
12/3/01 0 hr	1.49×10^6	6.8×10^3	4.0
12/4/01 24 hr	6.3×10^6	0	5.4 ?
12/5/01 48 hr	9.9×10^6	0	Meter faulty
12/6/01 72 hr	6.3×10^6	0	Meter faulty

? = Meter might have been faulty

When both organisms are inoculated into potato dextrose broth simultaneously, they both remain viable even though the pH drops from 5.0 to 3.62. Data shown in Table 4. show that when both organisms are inoculated into GABL supernatant (pH 4.0), only GABL grows.

Table 5. Results for *GABL* growth in *Pseudomonas aeruginosa* Supernatant (pH 6.7)

Time	Cfu/ml*	pH
O hr	5.65×10^7	6.7
24 hr	3.5×10^7	-
48 hr	3.1×10^7	-
72 hr	4.07×10^7	3.8
7 days	5.6×10^7	3.6

* Represents an average of three trials.

Table 6. Results for *GABS* growth in *Pseudomonas aeruginosa* Supernatant (pH 6.7)

Time	Cfu/ml*	pH
O hr	5.95×10^7	6.7
24 hr	$5.35x\ 10^7$	-
48 hr	4.69×10^7	-
72 hr	3.64×10^7	3.78
7 days	2.65×10^7	3.56

* Represents an average of two trials.

Table 7. Results for *Pseudomonas aeruginosa* growth in *GABL* Supernatant (pH 3.5)

Time	Cfu/ml*
O hr	4.0×10^5
24 hr	0
48 hr	0
72 hr	0

* Represents an average of two trials.

Table 8. Results for *Pseudomonas aeruginosa* growth in *GABL* Supernatant (pH adjusted to 7.0)

Time	Cfu/ml
O hr	3.3×10^7
24 hr	$+ 10^8$
48 hr	$+ 10^8$
72 hr	$+ 10^8$

Table No. 9: **Results for *Pseudomonas aeruginosa* growth in *GABS* Supernatant (pH 3.5)**

Time	Cfu/ml*
O hr	3.97×10^5
24 hr	0
48 hr	0
72 hr	0

* Represents an average of two trials.

Table 10. Results for *Pseudomonas aeruginosa* growth in *GABS* Supernatant (pH adjusted to 7.0)

Time	Cfu/ml
O hr	8.3×10^6
24 hr	$+ 10^8$
48 hr	$+ 10^8$
72 hr	$+ 10^8$

P. aeruginosa did not grow in GABL supernatant (pH 3.5), but grew in GABL supernatant (pH adjusted 7.0). These results are similar to that for GABS (Tables 9 and 10).

Table 11. Results for *GABL* growth on Emulsion Component Aerosol A 102

Date/Time	Cfu/ml
11/27/01 0 hr	1.55×10^6
11/28/01 24 hr	4.9×10^6
11/29/01 48 hr	4.3×10^6
11/30/01 72 hr	4.7×10^6
12/04/01 week 1	4.6×10^6
12/11/01 week 2	5.8×10^6
1/08/02 week 5	1.4×10^5

Table 12. Results for *GABL* growth on Emulsion Component Polystep B-27

Date/Time	Cfu/ml
11/27/01 0 hr	2.32×10^5
11/28/01 24 hr	1.52×10^5
11/29/01 48 hr	1.63×10^5
11/30/01 72 hr	1.05×10^5
12/04/01 week 1	1.16×10^4
12/11/01 week 2	9.0×10^2
1/08/02 week 5	0

As of week 5 of the study, *GABL* cell counts in Emulsion component Aerosol A 102 have decreased slightly (Table 11). However In Emulsion component polystep B-27,

GABL cell are not detectable. The cell counts started dropping at around week 1 and were completely dead by week 5. These results are consistent with *GABS* results growing in the same components.

Table 13. Results for *GABS* growth on Emulsion Component Aerosol A 102

Date/Time	Cfu/ml
11/27/01 0 hr	9.9×10^5
11/28/01 24 hr	9.3×10^5
11/29/01 48 hr	8.3×10^5
11/30/01 72 hr	6.2×10^5
12/04/01 week 1	5.5×10^5
12/11/01 week 2	5.6×10^5
1/08/02 week 5	3.7×10^3

Table 14. Results for *GABS* growth on Emulsion Component Polystep B-27

Date/Time	Cfu/ml
11/27/01 0 hr	5.4×10^4
11/28/01 24 hr	7.1×10^4
11/29/01 48 hr	7.0×10^4
11/30/01 72 hr	9.8×10^4
12/04/01 7 days	1.58×10^4
12/11/01 week 2	6.0×10^2
1/08/02 week 5	0

These experiments are **on-going**. As of week five, GABS cell count in Aerosol A-102 have decreased to lower that the initial inoculum. However, GABS in Polystep B-27, cell counts decreased after week one and were below our detection limits in five weeks. These results are similar to GABL results.

Refer to second quarter progress report protocol II (protocol for the growth-challenge experiments). No modifications to this protocol were made.

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Table 15. Results for *Candida tropicalis* growth on Emulsion Component Aerosol A-102

Date/Time	Cfu/ml
11/19/01 0 hr	5.3×10^5
11/20/01 24 hr	2.1×10^5
11/21/01 48 hr	3.0×10^5
11/23/01 72 hr	3.5×10^5
11/26/01 7 days	4.0×10^5
12/03/01 week 2	7.2×10^5
12/10/01 week 3	7.0×10^5
1/08/02 week 5	5.0×10^5

Table 16. Results for *Candida tropicalis* growth on Emulsion Component Polystep B-27

Date/Time	Cfu/ml
11/19/01 0 hr	3.0×10^5
11/20/01 24 hr	3.2×10^5
11/21/01 48 hr	3.6×10^5
11/23/01 72 hr	3.0×10^5
11/26/01 7 days	3.2×10^5
12/03/01 week 2	3.7×10^5
12/10/01 week 3	3.9×10^5
1/08/02 week 5	3.3×10^5

As of week 5 of the study, *Candida tropicalis* cell counts have neither increased nor decreased significantly for both components. This indicates that this organism is able to survive in these components. We cannot however conclude that the organism is able to grow in these components since the cell count has not increased significantly..

V. INTERPRETATION AND SIGNIFICANCE

Several observations are relevant. GABL and GABS can remain viable in Airflex 400 and in Airflex 192. However, our data do not support growth in those emulsions. One

major drawback, we **began these studies with a sample volume of 500 ml.** We have now corrected this flaw, and are using **a volume of l liter.** Furthermore, Dr. Schork's group can only carry out his analyses on **two samples/week.** Similar results were obtained wen GABL and GABS were inoculated in emulsion components (Aerosol A-102, and Polystep B-27). Our grow experiment using **supernatants (pH adjusted over a wide range of values)** suggest that acidity produced by GABL and GABS is responsibility for inhibition of *P.aeruginosa.*

VI. Conclusions and Recommendations

Results submitted in this report suggest that we need to **conduct separate growth experiments, and generate "growth curves" for GABL, GABS, *P. aeruginosa,* and *Candida tropicalis*** . Also, it is possible that GABL, GABS may be releasing **"lytic bacteriophages" and/or "bacteriocins".** I recommend that we design experiments to confirm or eliminate these substances as possible inhibitory agents.

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SECOND QUARTER PROGRESS REPORT

(SECOND-YEAR GRANT PERIOD)
February, 2002 - April, 2002

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIOLNS: BEHAVIOR OF MICROOORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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Edmonds, P., Second-Year Grant Period (Second Quarter Report, 5/8/02)

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I. EXECUTIVE SUMMARY

This reports contains experimental work performed during a short 2-month time period in an attempt to provide the next two Quarterly Reports on schedule. Research investigations were performed on a total of **10 different experiments.** Some of these experiments extend over a long - period with one sample/week for a 1-month; and held for 4 months before taking final samples. Other experiments were "modified" or "initiated" following discussions with the Program Manager (Dr. John Rabasco). Two organisms (***Gluconoacetobacter liquefaciens* [GABL]) and C*andida tropicalis***) were tested for growth in Airflex 400 (before biocide) and Airflex 192 (before biocide). After 4-weeks, GABL's population remained approximately the same. Cell counts for *Candida tropicalis* showed a slight increase. Samples will be taken again from both emulsions at the end of May. **Note: Dr. Joseph Schork's results on physical changes in these samples are inlcluded separately (Appendix).**

Three organisms (GABL, *Candida tropicalis*, and *Pseudomonas aeruginosa*) were tested separately for their ability to grow on each of four emulsion components: **Aerosol 102; Polystep B27; Airvol 205 (A solid); and Igepal CO-887 (a liquid).** Results from these studies show that two organisms (GABL and *Pseudomonas aeruginosa*) grew slightly on three of the emulsion components, and both organism showed **no growth in Polystep B27.** *Candida tropicalis* grew approximately two logs in each of the four emulsion components.

One on-going experiment involves testing the **cell-free extract** from growth of *Pseudomonas aeruginosa* in Potato Dextrose Broth against GABL. This experiment

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involve "filtration" and "concentrating" the cell-free extract. **No definitive results are available.**

Another "new" experiment involves determining **Growth Curves for GABL in Airflex 400 at two different temperatures: room and 37 C. (Note: This experiment is being repeated and modified due to sampling error.)**

II. OBJECTIVES OF EXPERIMENTS IN THIS REPORT

A. To perform **"growth measurements"** using GABL and *Candida tropicalis* inoculated "separately" into Airflex 400 and Airflex 192 for the purpose of generating data that will help to determine if these organisms are contributing to biodeterioration processes (i.e., chemical and physical changes) in these emulsions. **(Note: Physical parameters are measured by Dr. Joseph Schork, School of Chemical Engineering.)**

B. To perform **"growth measurements" (challenge experiments) using Emulsion usingEmulsionComponents** (added to a mineral salts medium) inoculated with different organisms (GABL, *Candida tropicalis* and *Pseudomonas aeruginosa*) to determine if any of these organisms could metabolize (utilize) an emulsion component (Aerosol 102; Polystep B27; Airvol 205; or Lgepal CO-887; and indirectly cause biodeterioration processes in emulsions that contain these components.

C. To test cell-free extract from *Pseudomonas aeruginosa* (following growth in Potato Dextrose Broth) for its ability to inhibit grow of GABL. These data will help explain a "potential" mechanisms that are involved in inhibitory actions of *Pseudomonas aeruginosa* against GABL in previously used "cross-streak" experiments on Agar Plates.

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D. To develop a Growth Curve for GABL in Airflex 400, from which will generate data relative to "inoculum size", and rate of growth at different temperatures; both of which are important to understanding biodeterioration processes.

III. METHODS/PROCEDURES/PROTOCOLS

A. Protocols for **"Growth of Studies"** to determine if organisms grow (increase in numbers) were described in the **"Third Quarter Report, First-Year Grant Period"**, but modified to "increase the volume of emulsion used (Airflex 400 and Airflex 192) in each experiment from 500 ml to 1,000 ml to prevent "using up the emulsion" before the end of the longer-sampling period: one sample/week for one month, and a final sample at the end of four months.

B. Protocols for **"challenge experiments"** to determine if organisms will metabolize (grow) on emulsion components were described in the **Second Quarter Report, First-Year Grant Period.**

C. Protocol used to determine a **"Growth Curve"** for GABL in Airflex 400 utilized a conventional **Tube-Dilution "Spread-Plate Method"** using Potato Dextrose Agar.

D. Protocol used to determine inhibitory action of **Cell-Free Extracts** from *Pseudomonas aeruginosa* growth in Potato Dextrose Broth involved the following: (a) filtration using a 0.22-um (pore size filter) to remove cells, (b) ammonium sulfate precipitation of crude extract, and (c) a critical point two-fold tube-dilution series, from which a drop from each concentration was added to a fresh inoculated plate (lawn) of GABL on Potato Dextrose Agar. The reiprocal of the highest dilution showing inhibitory activity is considered the end-point.

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IV. RESULTS

Results from GABL's growth in Airflex 400 (before biocide), and in Airflex 192 (before biocide).

These experiments were performed by inoculating GABL into1 liter of each emulsion to enable sampling for a longer period. One sample/week (for a period of 4 weeks) was removed from each emulsion sample and analyzed for microbial growth, and for physical and chemical changes by Dr. Schork's Group. These emulsion will be held under the same conditions for 4 months; after which the last samples will be analyzed. The results of GABL in Airflex 400 are shown in Table 1, and for Airflex 192 are shown in Table 2.

Table 1. **Results for *GABL* growth in Airflex 400, before biocide - (Latex 4)***

Date/Week	**Average cfu/ml**	**Color change**	**Odor change**	**Phase separation**
1/31/02, Time 0 before inoculation	0	None	None	None
2/4/02, week 1	3.0×10^6	None	None	None
2/11/02, week 2	6.3×10^6	None	None	None
2/18/02, week 3	7.3×10^6	None	None	None
2/25/02, week 4	3.0×10^6	None	None	None
End of May (4^{th} month)				

***Term in parenthesis denotes "label" used in Dr. Schork's Report.**

Table 2. **Results for *GABL* growth in Airflex 192, before biocide (Latex 5)***

Date/Week	**Average cfu/ml**	**Color change**	**Odor change**	**Phase separation**
1/31/02, Time 0 before inoculation	0	None	None	None
2/4/02, week 1	6.5×10^3	None	None	None
2/11/02, week 2	7.7×10^4	None	None	None
2/18/02, week 3	1.9×10^5	None	None	None
2/25/02, week 4	3.0×10^5	None	None	None
End of May (4^{th} month)				

*Term in parenthesis indicates the "label" used in Dr. Schork's Report.

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GABL's cell numbers remained relatively constant in samples of Airflex 400. However, *GABL* appears to be growing slightly in Airflex 192. No visual qualitative changes were observed in samples from either emulsion.

Results from *Candida tropicalis* growth in Airflex 400 (before biocide), and in Airflex 192 (before biocide).

These experiments were performed by inoculating *Candida tropicalis* into 1 liter of each emulsion to enable sampling for a longer period. The results from Airflex 400 (before biocide) are shown in Table 3, and results from Airflex 192 (before biocide) are shown in Table 4.

Table 3. **Results for *Candida tropicalis* growth in Airflex 400 – (Latex 6)***

Date/Week	Average cfu/ml	pH	Color change	Odor change	Phase separation
3/4/02, Time 0 before inoculation	0		None	None	None
3/4/02, Time 0 after inoculation	2.1×10^2		None	None	None
3/11/02, week 1	0		None	None	None
3/18/02, week 2	0		None	None	None
3/25/02, week 3	0		None	None	None
4/01/02, reset	9.8×10^3		None	None	None
4/02/02, 24 hr	0		None	None	None
4/04/02, 72 hr	0		None	None	None

*Term in parenthesis indicate the "label" used in Dr. Schork's Report.

Table 4. **Results for *Candida tropicalis* growth on Airflex 192, before biocide – (Latex 7)***

Date/Week	Average cfu/ml	pH	Color change	Odor change	Phase separation
3/4/02, Time 0 before inoculation	0		None	None	None
3/4/02, Time 0 after inoculation	2.8×10^2		None	None	None
3/11/02, week 1	0		None	None	None
3/18/02, week 2	3.0×10^4		None	None	None
3/25/02, week 3	3.1×10^4		None	None	None
4/01/02, week 4	1.4×10^4	4.5	None	None	None
4/08/02, week 5	3.0×10^3	4.5	None	None	None
4/15/02, week 6	8.0×10^2	4.5	None	None	None
4/22/02, week 7	0	4.5	None	None	None
4/29/02, week 8	0		None	None	None

*Term in parenthesis indicate the "label" used in Dr. Schork's Report.

Candida tropicalis cells were not detected (i.e., cfu/ml on agar plate) from samples of Airflex 400 during the three-week period. These results were unexpected. The emulsion was re-inoculated (with an inoculum size higher than at "Time zero"), and no cells were detected in samples after 24 hours. These results are shown in Table 3. However, this organism survived and showed a slight increased in numbers from samples taken from Airflex 192 over a period of 4 weeks These data are shown in Table 4.

Results of GABL's growth using four emulsion components (Aerosol 102, Polystep B27, Airvol 205, and Igepal CO-887).

GABL was inoculated into a sterile Mineral Salts Medium to which each emulsion component was added (separately) as a "supplement". Then, growth was measured on Potato Dextrose Agar, using the Spread-Plate Method. Table 5 shows data from samples that contain Aerosol 102 and Polystep B27. Cell numbers of GABL increased approximately three logs in Aerosol 102. However, cells in Polystep B27 were not detected after 24 hours. This experiment will be "repeated".

Table 5. **Results of *GABL's* growth using Aerosol 102 and Polystep B27**

Date / Time	Aerosol 102 (Cfu/ml)	Polystep B27 (Cfu/ml)
3/12/02, Time 0	4.6×10^3	4.2×10^3 (colonies unusually small)
3/13/02, 24 hr	4.6×10^3	1.6×10^3 (colonies unusually small)
3/14/02, 48 hr	1.5×10^5	0
3/15/02, 72 hr	3.2×10^6	0
3/19/02, week 1	3.0×10^6	0
3/26/02, week 2	3.3×10^6	0 (last sample)
4/02/02, week 3	8.7×10^5	

Table 6 shows data from samples containing Airvol 205 and Igepal CO-887. GABL's cell numbers increased in samples from Airvol 205. Unexplained contamination was detected in Igepal CO-887 after 48 hrs. Another sample (containing this component) was re-inoculated with GABL (using an inoculum size similar that of "Time Zero"). GABL's cell numbers increased approximately two logs in 72 hrs.

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Table 6. **Resulst of *GABL's* growth using Airvol 205 and Igepal CO-887**

Date / Time	Cfu/ml on Airvol 205	Cfu/ml on Igepal CO-887
3/12/02, Time 0	6.3×10^3	5.2×10^3
3/13/02, 24 hr	8.5×10^3	1.18×10^4
3/14/02, 48 hr	3.0×10^4	8.1×10^4 * (contaminated)
3/15/02, 72 hr	2.16×10^5	5.4×10^3 (Reset) (Time 0)
3/19/02, week 1	3.0×10^6	1.39×10^4 (24 hr)
3/26/02, week 2	3.5×10^6	5.3×10^5 (48 hr)
4/02/02, week 3	6.5×10^5	1.16×10^6 (72 hr)

Results of *Candida tropicalis'* growth using four emulsion components (Aerosol 102, Polystep B27, Airvol 205 and Igepal CO-887).

Candida tropicalis was inoculated into a sterile Mineral Salts Medium to which each emulsion component was added (separately) as a "supplement". Then, growth was measured on Potato Dextrose Agar, using the Spread-plate Method. Results in Table 7 show cell numbers for this organism in samples supplemented with Aerosol 102 and Polystep B27. Growth increased slowly by approximately three logs at the end of a two-week period. Results in Table 8 show similar growth patterns for *Candida tropicalis* in samples that contained Airvol 205 and Igepal CO-887.

Table 7. **Results of *Candida tropicalis* growth using Aerosol 102 and Polystep B27**

Date / Time	Aerosol 102 (Cfu/ml)	Polystep B27 (Cfu/ml)
3/25/02, Time 0	2.0×10^2	2.1×10^2
3/26/02, 24 hr	4.7×10^2	1.5×10^3
3/27/02, 48 hr	7.2×10^3	7.5×10^3
3/28/02, 72 hr	5.5×10^4	3.0×10^4
4/03/02, week 1	3.3×10^5	1.1×10^5
4/11/02, week 2	4.3×10^5	2.0×10^5

Table 8. **Results of *Candida tropicalis* growth on Airvol 205 and Igepal CO-887**

Date / Time	Cfu/ml on Airvol 205	Cfu/ml on Igepal CO-887
3/25/02, Time 0	1.9×10^2	1.3×10^2
3/26/02, 24 hr	6.9×10^3	3.0×10^3
3/27/02, 48 hr	2.9×10^5	8.3×10^4
3/28/02, 72 hr	3.1×10^5	2.6×10^5
4/03/02, week 1	3.0×10^5	5.9×10^5
4/11/02, week 2	5.1×10^5	7.2×10^5

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Results of *Pseudomonas aeruginosa* growth using four emulsion components (Aerosol 102, Polystep B27, Airvol 205, and Igepal CO-887).

Pseudomonas aeruginosa cells were inoculated into a sterile Mineral Salts Medium to which each emulsion component was added (separately) as a supplement. Then, growth was measured on Potato Dextrose Agar, using the Spread-Plate Method. Table 9 shows data from samples that contain Aerosol 102 and Polystep B27. Table 9 shows that cell numbers for this organism increased by two logs in samples that contained Aerosol 102. However, *Pseudomonas aeruginosa* cells were not detected in samples that contained Polystep B27 after 24 hrs. **This experiment is being repeated.**

Table 9. **Growth Response of *Pseudomonas aeruginosa* using Aerosol 102 and Polystep B27**

Date / Time	**Aerosol 102 (Cfu/ml)**	**Polystep B27 (Cfu/ml)**
4/01/02, Time 0	9.9×10^3	9.2×10^3
4/02/02, 24 hr	8.8×10^3	0
4/03/02, 48 hr	8.6×10^3	0
4/04/02, 72 hr	1.4×10^5	0
4/11/02, week 1	8.0×10^4	stopped
4/18/02, week 2	5.0×10^4	

Table No. 12 *Pseudomonas aeruginosa* **growth on Airvol 205 and Igepal CO-887**

Date / Time	**Cfu/ml on Airvol 205**	**Cfu/ml on Igepal CO-887**
4/01/02, Time 0	8.9×10^3	9.3×10^3
4/02/02, 24 hr	2.4×10^6	7.8×10^6
4/03/02, 48 hr	3.3×10^7	1.0×10^8
4/04/02, 72 hr	1.8×10^8	1.0×10^8
4/11/02, week 1	6.0×10^7	5.6×10^7
4/18/02, week 2	4.5×10^6	3.7×10^7

V. INTERPRETATION AND SIGNIFICANCE

From data shown in Table 1 and Table 2, *Gluconoacetobacter liquefaciens* (GABL) remains viable, but do not increase in numbers in Airflex 400 (before biocide) at room temperature. A similar growth response was exhibited by GABL in Airflex 192 (before biocide). Additional information that will be generated from the development of a Growth Curve for GABL in Airflex 400 may help to understand this grow response (**Note: The Growth Curve experiment has been planned but not started.)**

Growth response data shown in Table 3 and Table 4 using *Candida tropicalis* in Airflex 400 (before biocide) and Airflex 192 (before biocide) suggest that this organism dose not survive in Airflex 400, but survive for several weeks in Airflex 192. **These experiments need to be repeated (in duplicate using both a small and a relative large inoculum size.**

When the three test organisms (GABL, *Candida tropicalis* and *Pseudomonas aeruginosa*) were evaluated against four emulsion components. Only *Candida tropicalis* survived or grew on all four components. GABL and *Pseudomonas aeruginosa* survived or grew slignhtly on three component (Aerosol 102, Airvol 205, and Igepal CO-887). Growth response was poor in Polysstep B27 for these two organisms. **Does Polystep B27 contain inhibitory properties toward these bacteria? I will repeat this experiment to rule out technical error.**

VI. CONCLUSIONS AND RECOMMENDATIONS

The unexplainable results for the sudden decline of *Candida tropicalis* in Airflex 400 (Table 3), may be due to inoculum size and/or age of culture from which the inoculum was prepared. **This experiment will be repeated.**

It may be significant to evaluate Polystep B27 for inhibitory activity against GABL and *Pseudomonas aeruginosa*.

I recommend that "high-priority" be placed on **two planned experiments:**

(i) Determination of a Growth Curve for GABL in Airflex 400 at two temperatures (Room and 37 C). For comparison the Growth Curve should be performed at both temperatures using GABL in Potato Dextrose Broth.

(ii) Continue (i.e., intensify) the work on experiments to determine if "crude cell-free extracts" from *Pseudomonas aeruginosa* exhibit inhibitory action against GABL.

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THIRD QUARTER PROGRESS REPORT

(SECOND-YEAR GRANT PERIOD)
May 9, 2002 - July 18, 2002

PROJECT NO: 3206634

MICROBIOLOGY OF POLYMER EMULSIONS: BEHAVIOR OF MICROORGANISMS AND BIOCIDES IN THE EMULSION ENVIRONMENT

BY

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Edmonds, P., Second-Year Grant Period (Third Quarter Report, 7/24/02)

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Edmonds, P., Second-Year Grant Period (Third Quarter Report, July 24, 2002)

I. EXECUTIVE SUMMARY

The focus of research described in this report is on two broad groups of experiments: (i) the determination of a **growth curve** for *Gluconoacetobacter liquefaciens* (GABL) in Airflex 400, and (ii) **new experiments** that will help us to describe more clearly the nature of the "substance" produced by *Pseudomonas aeruginosa* that inhibits the growth of GABL and other organisms that have been isolated from contaminated emulsions.

Determination of a growth curve for GABL. First, we conducted a **"control experiment"** by determining a **growth curve** for GABL growing in Potato Dextrose Broth incubated at two different temperatures: room and at 37°C. Samples were processed at specific time periods using the conventional **Tube-Dilution Spread-Plate method**, and by **Turbidity Measurements (%T)**, using a spectrophotometer. Data collected by each method was plotted on separate graphs. Turbidity (%T) is an indirect measure of growth, and values result from the presence of both viable and dead cells. Results obtained from the Tube-Dilution Spread-Plate Method, measures **only viable cell numbers,** and are expressed as **colony forming units (CFU/ml).** When both sets of data are plotted on the same graph, a specific %T value can be correlated to a specific number of viable cells (CFU/ml). Our turbidity value of 96%T in Fig.1 **(time zero),** correlates with 8.1 X 10^6 CFU/ml as the **initial inoculation size (Table 1).** It is important to remember that cell numbers below 1 X 10^4 CFU/ml cannot be measured accurately using

turbidity. GABL's growth (CFU/ml) in potato destrose broth, incubated at both temperatures were similar for 12 days (Fig. 2).

We determined **Growth Curves for GABL in Airflex 400**, using four variables: (i) GABL inoculated in the concentrated emulsion (100% Airflex 400) and incubated at room temperature, (ii) GABL inoculated in the concentrated emulsion (100% Airflex 400) and incubated at 37°C, (iii) GABL inoculated in diluted emulsion (75% Airflex 400+25% sterile water) and incubated at room temperature, and (iv) GABL inoculated in the diluted emulsion (75% Airflex 400+25% sterile water) and incubated at 37°C. Growth measurements in these experiments were determined using only the Tube Dilution Spread-Plate Method.

Growth curves for GABL growing in 100% Airflex 400 at both temperatures are shown in Figure 3. GABL exhibited better growth at room temperature. The **lag phase** was approximately 3 days, and the **exponential phase** lasted approximately 9 days. The **exponential phase** for GABL growing at 37°C lasted approximately 3 days.

Growth curves for GABL in the diluted emulsion (75% Airflex 400 + 25% sterile water), and incubated at both temperatures are shown in Fig. 4. The **exponential phase** for cells grown at room temperature (with an intermittent deline) lasted approximately 12 days. **GABL's best growth response occurred in concentrated Airflex 400 incubated at room temperature. Using these conditions, GABL's growth rate (two different periods) were 0.60 day, and 1.97 days, respectively.**

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We have begun experiments aimed at generating "new data" on the **substance** produced by *Pseudomonas aeruginosa* that exhibits GABL and some other organisms that have been isolated from contaminated emulsions. First, "cell-free extracts" were processed by centrifugation to remove cells, and the supernatant was subsequently vacuum-pulled through filters (0.22 um pore size). This extract was diluted 10-fold, and added drop-wise to the surface of potato dextrose agar plates (a lawn) of GABL, and incubated for 5 days at room temperature. Results: no inhibition. Other experiments consisted of using two preparations: a) two-fold dilutions of the cell-free extract, and b) using two-fold dilutions of chloroform-treated *Pseudomonas aeruginosa* "cell-free" extract. Two-fold dilutions of both preparations were added drop-wise to the surface of potato dextrose agar plates: one set of plates were inoculated with GABL, and the other set of plates were inoculated *P. aeruginosa*.

Results from those experiments showed **"inhibitory action"** (pitted halos) on *P. aeruginosa* plates, and "clear-zones" of no growth on plates inoculated with either GABL or *P. aeruginosa.* **Efforts to reproduce these results have been irregular.** These observations are indicative of a **lysogenic** *P.aeruginosa* culture (i.e., contain phages with their DNA integrated into the bacterial chromosome). Spontaneously, such "phages" are released. Alternatively, such actions may result from "bacteriocins" or other "antibacterial peptides "secreted" by *P.aeruginosa.* Such actions among microorganisms are discussed in the **"INTERPRETATION AND SIGNIFICANCE"** SECTION of this report.

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II. OBJECTIVES OF EXPERIMENTS DESCRIBED IN THIS REPORT

A. To determine a "growth curve" for *Gluconoacetobacter liquefaciens* (GABL) in Airflex 400. This experiment will be conducted using the following variables: i) "initial inoculation sizes", ii) two different emulsion concentrations: 100% Airflex 400, and diluted emulsion (75% Airflex 400 + 25% sterile water), and iii) two different incubation temperatures (Room and 37°C). Data generated from these experiments will enable us to describe GABL's "lag Phase", "exponential Phase", and rate of growth; all of which will contribute to our understanding of biodeterioration processes.

B. To determine a growth-curve **"Control Experiment"**, using *Gluconoacetobacter liquefaciens* (GABL) in Potato Dextrose Broth, incubated at two different temperatures (Room and 37°C). This experiment will establishment "base-line" data for comparison to GABL's growth response in Airflex 400.

C. To **expand** our experimental work that deal with evaluating "cell-free" extracts from *Pseudomonas aeruginosa* (following growth in Potato Dextrose Broth) for its ability to inhibit growth of *Gluconoacetobacter liquefaciens* (GABL). Specific aims of these experiments are to generate data that may help to explain a "potential" mechanisms that is responsible for the inhibitory actions that were observed previously, when using "cross-streak" experiments on agar plates.

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III. METHODS/PROCEDURES/PROTOCOLS

A. Protocols for determining **"Growth Curves"** for GABL in Airflex 400 and/or "control experiments" utilized the conventional **Tube-Dilution "Spread-Plate Method" using Potato Dextrose Agar.**

B. Protocols for determining inhibitory action of **cell-free extracts** from *P. aeruginosa* on GABL were described in the **Second Quarter Report, 5/8/02.**

IV. RESULTS

A major objective of research described in this report was to determine a growth curve for GABL in Airflex 400. First, we developed **"base line data" in a CONTROL EXPERIMENT** from GABL growing in Potato Dextrose Broth.

All cultures used in these experiments were performed using a pure culture of GABL that is maintained in separate vials of potato dextrose broth +15% glycerol in a Revco Freezer at -80°C. A sterile glass pasteur pipet was used to remove a portion of GABL's frozen culture from its vial, and sub-cultured by streaking two potato dextrose agar plates in a manner to obtain isolated colonies. One of these plates was incubated at room temperature, and the other plate was incubated at 37°C. When visible colonies were observed on the plates (usually within 2 to 5 days), Gram-stained smears were prepared and examined microscopically to ascertain purity.

Then a suspension of GABL's cells was prepared in sterile potato dextrose broth, and standardized, using a spectrophotometer with wavelength set at 600 nm, by adjusting its turbidity to 95%T.

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Then, 10 ml of this standardized cell suspension was used to inoculate each of two flasks separately that contained 250 ml of potato dextrose broth. One flask was incubated at room temperature, and the other flask was incubated at 37°C.

To determine a growth curve, samples were removed (aseptically) from each flask immediately following inoculation **(zero time),** and at subsequent intervals. One portion of each sample was processed using the conventional **Tube-Dilution Spread-Plate Method,** and a separate portion of each sample was used to take turbidity measurements (%Transmission). These data are shown in Table 1, and viable plate counts (CFU/ml) are shown in Table 2. Subsequently, these data were used to plot the Growth curves: the graph for turbidity measurements is shown in Figure 1, and the graph for viable cell counts (CFU/ml are shown in Figure 2. Turbidity is an indirect measurement of growth, and the %T values result from the presence of both viable and dead cells. Results obtained from the Tube-Dilution Spread-Plate Method measures **only viable cells,** and are expressed as **colony forming units (CFU/ml).** When both sets of data are plotted on the same graph, a specific %T value can be correlated to a specific number of cells (CFU/ml). During growth, cell numbers below 1 X 10^4 CFU/ml, usually cannot be measured by turbidity (%T). Our turbidity measurements (Table 1) show that a 96%T value correlates with 8.1 X 10^6 CFU/ml of GABL in the initial inoculation size (**time zero)** in the flask that was incubated at room temperature. Cell numbers (CFU/ml) from sampling of GABL in flasks incubated at room temperature were used to develop the **growth curve** for GABL (Figure 2).

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Table 1. Turbidity measurements for GABL in Potato Dextrose Broth (%T)

Time (days)		Rm Temp	37*C
0	(5/16/02)	96	96
1	(5/17/02)	94	91.5
7	(5/23/02)	43.1	21
8	(5/24/02)	41	19
12	(5/28/02)	37.5	16.1

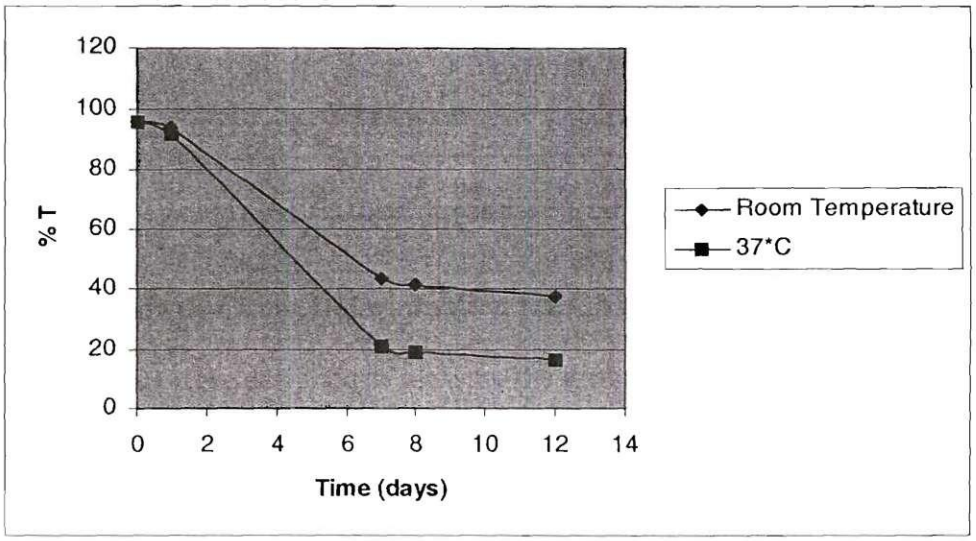

Figure 1. Growth-curve turbidity measurements (%T) for GABL in Potato Dextrose Broth.

Table 2. Spread-plate measurements for GABL on Potato Dextrose Agar (CFU/ml)

Time (days)		Rm Temp	37*C
0	(5/16/02)	8.10E+06	7.80E+06
1	(5/17/02)	1.42E+07	1.01E+07
7	(5/23/02)	1.01E+08	5.70E+07
12	(5/28/02)	8.40E+06	4.40E+06
15	(5/31/02)	6.90E+06	1.38E+06

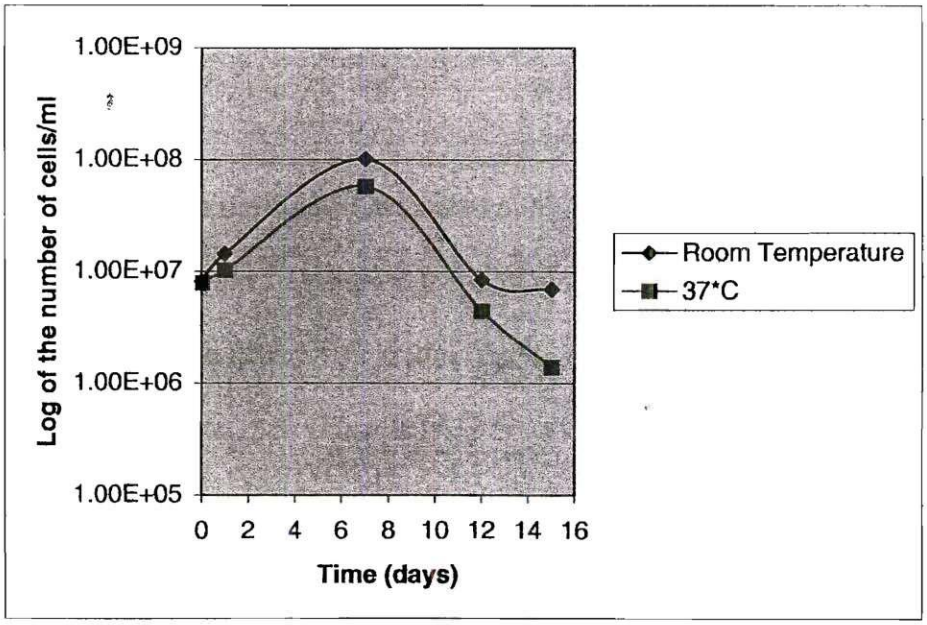

Figure 2. The growth curve for GABL in potato dextrose broth (CFU/ml).

Growth-Curve Determination for GABL Growing in Airflex 400.

Experiments were designed to measure the growth response of GABL in Airflex 400, using four variables: (i) GABL inoculated in the concentrated emulsion (100% Airflex 400) and incubated at room temperature, (ii) GABL inoculated in the concentrated emulsion (100% Airflex 400) and incubated at 37°C, (iii) GABL inoculated in the diluted emulsion (75% Airflex 400 + 25% sterile water) and incubated at room temperature, and (iv) GABL inoculated in the diluted emulsion (75% Airflex 400 + 25% sterile water). Cell numbers (CFU/ml) obtained from GABL cultures growing under each of the four conditions, and processed by the Tube Dilution Spread-Plate Method are shown in Table 3.

The **growth curve** for GABL growing in 100% Airflex 400 is shown in Figure 3. The initial inoculation size for the culture incubated at room temperature was 3 X 10^5

CFU/ml. During the **lag phase** (approximately 3 days), cell numbers declined. The **exponential phase** lasted approximately 9 days, and GABL continued to grow during the entire sample period of 28 days. GABL's initial inoculation size and growth response in the **lag phase** of the culture incubated at 37°C were similar to those of GABL incubated at room temperature. However, the **exponential phase** (approximately 3 days) exhibited a greater **growth rate**, and a gradual decline for approximately 10 days, after which the cells continued to grow at a relatively constant rate for the duration of the sampling period of 28 days.

The **growth curve** for GABL in the diluted emulsion (Aiflex 400 +25% sterile water) is shown in Figure 4. The initial inoculation size for cultures incubated at room temperature and at 37°C were 3.4 X 10^6 CFU/ml and 3.6 X 10^5 CFU/ml, respectively. The **lag phase** for both lasted approximately two days. The **eponential phase** for cells growing at room temperature lasted approximately 12 days, with a decrease in cell numbers for 2 days (day 7 to day 10). The duration of the **exponential phase** for cells growing at 37°C was approximately three days; and no viable cell detected after 14 days.

When comparing GABL's growth response under variable conditions, the best growth occurred in the concentrated emulsion (100% Airflex 400) incubated at room temperature. Consequently, we calculated the generation time for GABL growing under these conditions. Cell numbers used for **calculating the generation time** were selected from two different intervals within the **exponential growth phase (See Table 3 and Figure 3),** and are shown below:

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Time Interval	CFU/ml	Generation Time (days)
June 11 - 13	9.30E+05 - 9.30E+04	0.60 (days)
June 7 - 10	3.40E+05 - 9.80E+05	1.98 (days)

Table 3. Growth response of GABL in Airflex 400 (CFU/ml)

Time (days)		100% Rm	100% 37°C	75% Rm	75% 37°C
0	(6/10/02)	3.05E+05	1.79E+05	3.40E+06	3.60E+05
1	11-Jun	9.30E+03	4.60E+04	4.30E+05	3.40E+04
3	13-Jun	9.30E+04	8.90E+03	1.92E+06	2.67E+04
7	17-Jun	3.40E+05	1.63E+07	4.20E+07	2.49E+07
10	20-Jun	9.80E+05	3.10E+06	3.40E+07	5.40E+07
14	24-Jun	8.90E+06	2.73E+05	7.70E+07	3.49E+04
17	27-Jun	6.50E+06	1.61E+04	3.03E+07	0
28	8-Jul	4.70E+07	4.60E+04	1.66E+05	0

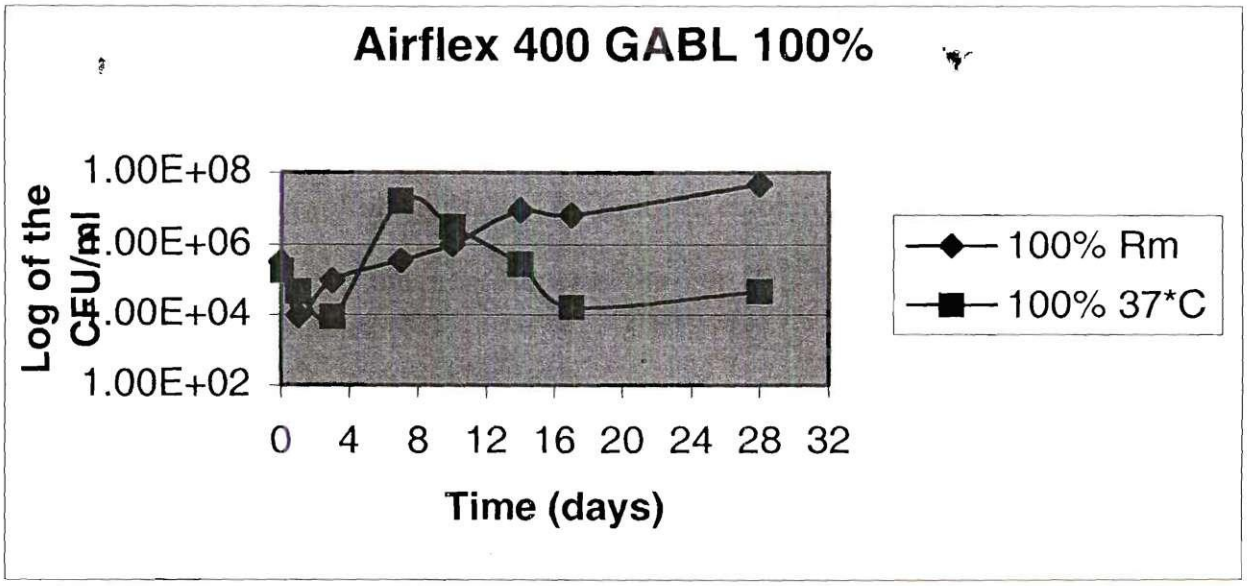

Figure 3. Growth response of GABL in concentrated Airflex 400 at two different temperatures.

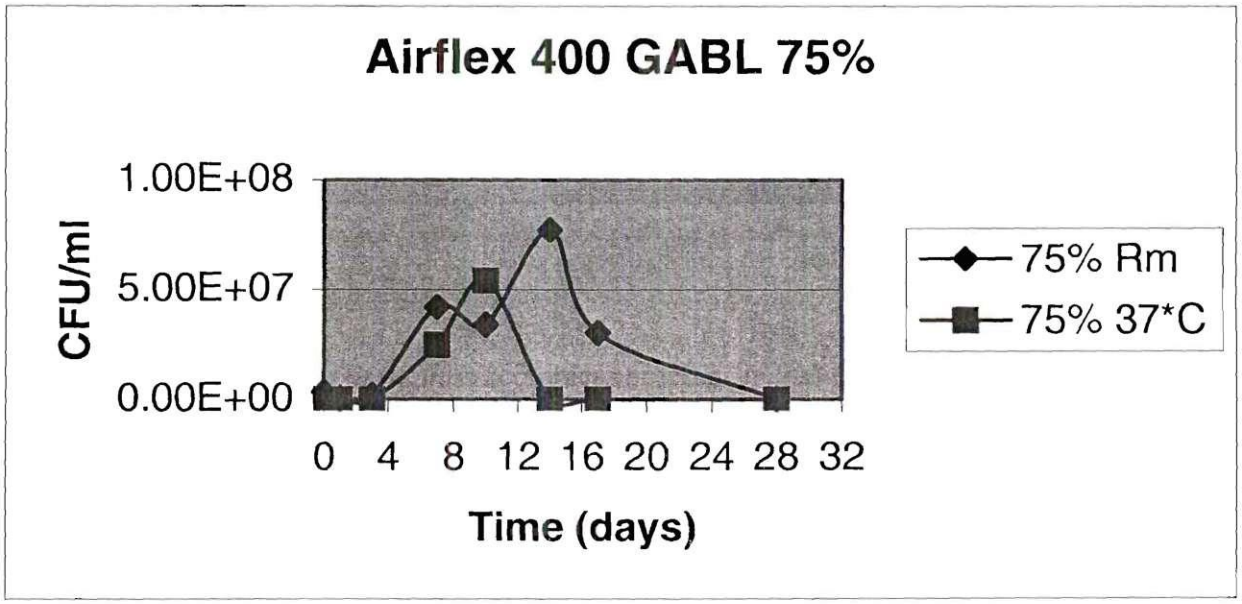

Figure 4. Growth response of GABL in diluted emulsion (Airflex 400 + 25% sterile water at two different temperatures.

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Our evaluation of "**inhibitory substances**" of *P. aeruginosa* show "halos" in the center of isolated colonies (Fig. 5), as compared to a normal culture in Fig. 6. "Phage-like" zones of growth inhibition can be seen in Fig. 7. Potato dextrose agar plates inoculated with a "lawn" of GABL (Fig 8a) to which chloroform-treated extracts were added, show areas of zone inhibition in the **center** of the drop (Fig. 8b).

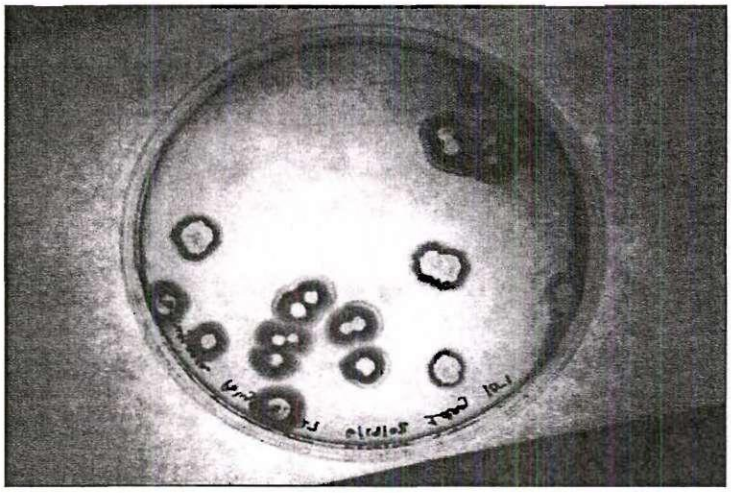

Figure 5. *Pseudomonas aeruginosa* colonies showing "halos" (zones of growth inhibition) in the center of the colonies.

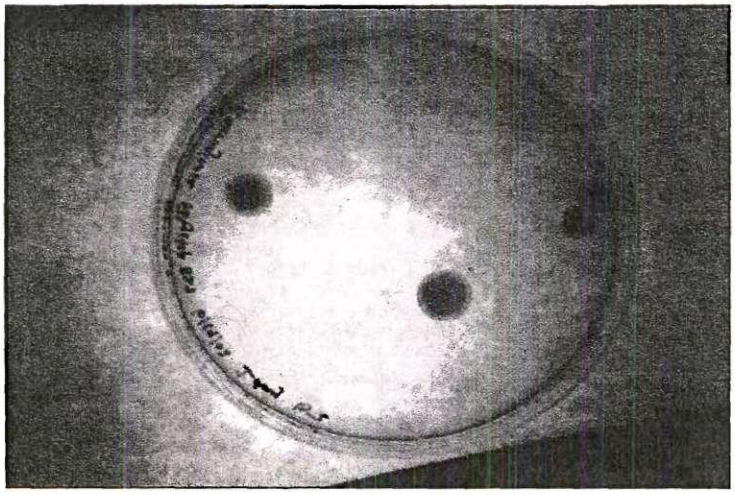

Figure 6. A potato dextrose agar plate showing "normal" *Pseudomonas aeruginosa* colonies.

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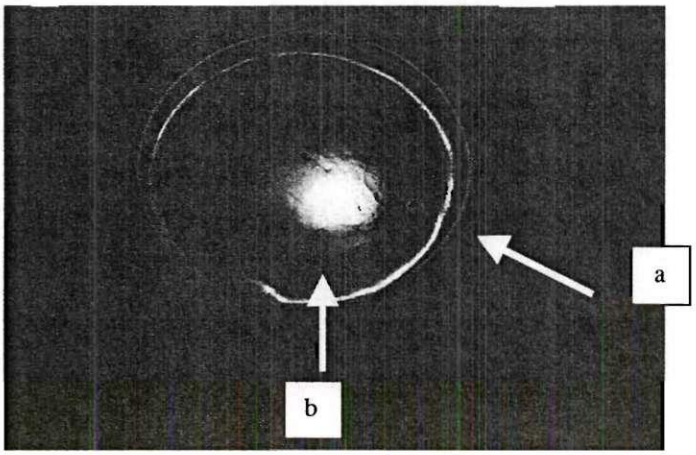


Figure 7. A *Pseudomonas aeruginosa* lawn on a potato dextrose agar plate to which drops of a chloroform-treated *P.aeruginosa* cell-free extracts have been added. And incubated for 24 hr at 37°C. "Pitted" areas of cell lysis are shown where drops (a) and (b) were added.

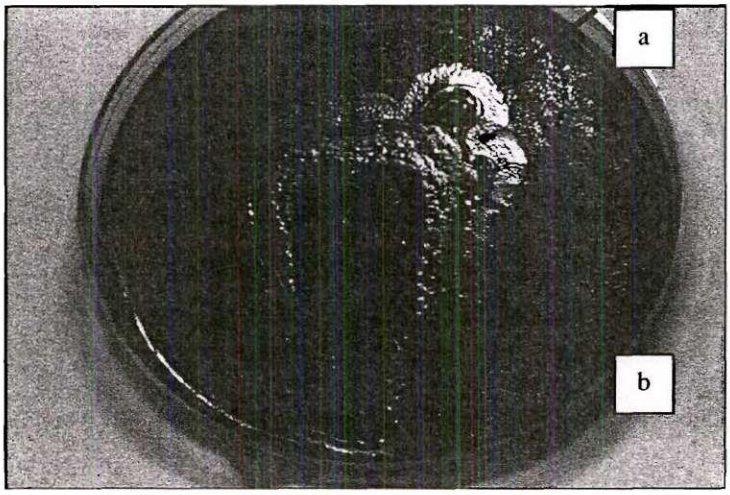
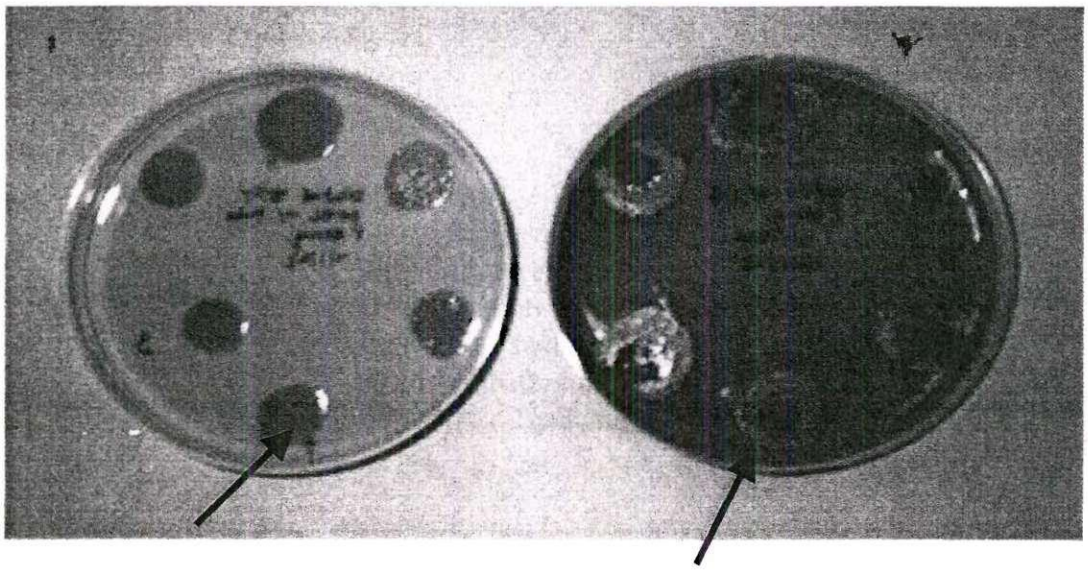


Figure 8. A potato dextrose agar plate inoculated with a lawn of GABL, to which a non-chloroform- treated drop of *P. aeruginosa*'s cell-free extract had been added, and incubated for 48 hours at 37°C.



A **B**

Figure 9. Plate (A) shows a potato dextrose agar plate inoculated with six drops of a "normal" (undulited) *P. aeruginosa* 24 hour broth culture. Plate (B) shows a potato dextrose agar plate to which six drops from a suspension (undiluted) of "pitted-halos" had been added.

V. INTERPRETATION AND SIGNIFICANCE

Our efforts to reproduce the activity of the "inhibitory substances" have been irregular. Consequently, we speculate that such activity may result from **prophages.** Evidence supporting Such activity have been reported for *Streptococcus pneumoniae* (4). The "pitted" halos, may Have resulted from **lytic phages**, as observed recently in *Burkholderia thailandensis* (5). Bacteriocins from *Bacillus cereus* (1) and *Listeria innocua* (3) have been considered for use as biopreservatives in food products for human consumption. Antimicrobial peptides are produced by *Propionibacterium jensenii* as an "inactive substance", but can be activated by protease treatment (2).

VI. CONCLUSIONS AND RECOMMENDATIONS

Results of experiments described in this report show that **GABL** grows well in concentrate Airflex 400, and grows less favorably in diluted Airflex 400. GABL's growth rate in Airflex 400 (calculated from two different periods) were 0.60 days, and 1.97 days, respectively.

Our data also show that "inhibitory substances" produced by *P. aeruginosa* exhibited "phage-like" activity, but also have similarities to "bacteriocins", and/or antimicrobial peptides that could potentially be "activated" in the emulsion environment. **Recommendations:** These data are interesting, and our preliminary "evidence" are similar to "inhibitory substances" recently reported in the literature that are being considered for use as **biopreservatives** to prevent or reduce contamination of foods for human consumption. It is reasonable, that such substances should be further characterized and considered for potential use as biodeterioration inhibitors for emulsions.

VII. REFERENCES

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